SRC FAMILY KINASE INVOLVEMENT IN SELECTED CANCER CELL PHENOTYPES

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ABSTRACT

The non-receptor tyrosine kinase Src has been found to be overexpressed and activated in many human cancers, where it has been implicated in changes in cellular proliferation, adhesion, migration, apoptosis, angiogenesis, and tumour growth. In addition, several other members of the Src family have also been implicated in various cancer phenotypes. Our examination of a wide panel of colon, breast, and lung cancer cell lines revealed that not only Src, but also Yes, Fyn, Lyn, and Lck, were expressed at both the mRNA and protein levels in different combinations, and at varying levels, between cell lines. When examined for kinase activity, it was discovered that only a subset of the expressed Src family members had detectable kinase activity within a given cell line. To investigate the involvement of the Src family members in the proliferation, adhesion, migration, and colony forming ability of four selected cancer cell lines, both Src family kinase inhibitors, which inhibit the kinase activity of multiple Src family members, and RNA interference, which selectively decreases the expression of individual proteins, were used. It was found that the involvement of these proteins in all of the cellular processes investigated was cell line-specific, with the greatest effects observed in HT29 cells, which have relatively high Src protein levels and kinase activity. Furthermore, the consequences of Src family member inhibition were also inhibitor specific, as treatment with PP2 and SKI I generally had greater effects on the cellular processes examined than did treatment with SU6656 or SKI II. It was also found that the inhibition of multiple Src family kinases by at least one of the inhibitors generally resulted in greater effects on the cancer cell phenotypes investigated than were observed when the expression of Src, Fyn, or Yes was decreased using RNA interference. This suggests that multiple Src family members may need to be targeted in order to inhibit the increased proliferation, cell-matrix adhesion, migration, and colony forming ability exhibited by cancer cells. The identification of the cancer cell phenotypes in which particular Src family members are involved is of interest, as these proteins are attractive targets for cancer therapy.

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5-FU	5-fluorouracil
Abl	c-Abl oncogene
ACF	aberrant crypt foci
APC	adenomatous polyposis coli
BCR	breakpoint cluster region
BRCA	Breast Cancer
CAMS	cell-cell adhesion molecules
CD44	cluster of differentiation 44
CDCP1	CUB-domain-containing protein 1
Cdk	cyclin dependent kinase
CML	chronic myelogenous leukemia
CSF-1	colony stimulating factor type 1
Csk	carboxy-terminal Src kinase
c-Src	cellular Src
CT	computed tomography
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	endothelial to mesenchymal transition
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FAP	familial adenomatous polyposis
FGF	fibroblast growth factor
FOBT	fecal occult blood testing
HNPCC	hereditary nonpolyposis colorectal cancer
hMLH1	human mutL homologue 1
hMSH2	human mutS homologue 2
HRP	horseradish peroxidase
IL	interleukin

Jak2	Janus Kinase 2
LPA	lysophophatidic acid
MAPK	mitogen activated protein kinase
MMR	mismatch repair
MOPS	N-morpholinopropanesulfonic acid
MTS	3-(4,5-dimethylthiazol-2-yl)- 5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSCLC	non small cell lung cancers
P13K	phosphoinositide 3'-kinase
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PP1	4-Amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
PP2	4-amino-5-(4-chorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
RNAi	RNA interference
RSV	Rous Sarcoma Virus
RTK	receptor tyrosine kinase
SH	Src homology
shRNA	small hairpin RNA
siRNA	small interfering RNA
SKI I	Src Kinase Inhibitor I
SKI II	Src Kinase Inhibitor II
SKI-606	4-Anilino-3-quinolinecarbonitrile
SU6656	2-Oxo-3(4,5,6,7-tetrahydro-1H-indol-2-ylmethylene)-2,3-dihydro-1H-ole-5-sulfonic acid dimethylamide
TNM	tumour, node, metastasis
Tyr	tyrosine
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
v-Src	viral Src

1. REVIEW OF THE LITERATURE

1.1. INTRODUCTION

The non-receptor tyrosine kinase Src has been implicated in a wide variety of cellular phenotypes and has been found to contribute to the development and progression of a large number of different cancers, including colon, breast, pancreatic, brain, lung, and ovarian cancers, as well as melanomas and hematopoietic cancers (Summy and Gallick, 2003; Lieu and Kopetz, 2010). In cancer cells, increased Src activity has been implicated in changes in proliferation, cellular adhesion, migration, apoptosis, angiogenesis, and tumour growth (Thomas and Brugge, 1997; Irby and Yeatman, 2000; Summy and Gallick, 2003, Yeatman, 2004; Lieu and Kopetz, 2010). However, Src is only the prototype member of the Src family of nine non-receptor tyrosine kinases. Some of the other Src family kinases (SFKs), notably Yes, Fyn, Lyn, and Hck, have also been implicated in the development and progression of human cancers (Lieu and Kopetz, 2010). As such, the Src family has become an attractive target in cancer therapy, and a number of Src-directed chemical inhibitors are either currently in use or are undergoing clinical trials for the treatment of both hematopoietic cancers and various solid cancers (Wheeler *et al*., 2009; Lieu and Kopetz, 2010). As multiple SFKs may be co-expressed within a given cell, it is of interest to determine what cellular events the individual SFKs are involved in. The goal of this work was therefore to investigate the involvement of the individual SFKs in cancer cell phenotypes in selected human cancer cell lines by using RNA interference (RNAi), as well as commercially available SFK inhibitors. The work presented herein provides further insight into the involvement of the ubiquitously expressed SFKs, Src, Yes and Fyn, in the proliferation, adhesion, migration and colony forming ability of selected cancer cell lines.

The following literature review will focus on the development and progression of cancer, with an emphasis on colon cancer, as well as the involvement of the SFKs in several cancer cell phenotypes. Chemical SFK inhibitors currently used in cancer therapy, as well as those undergoing clinical trials, will also be discussed.

1.2. CANCER

Cancer is a disease characterized by uncontrolled cell growth. This disease can develop in many different cell types; there are more than one hundred different cancers affecting different organs currently known. Furthermore, there may also be distinct subtypes of cancer within a given organ, each of which potentially requires a different form of treatment. This results in cancer being an extremely complex disease both in its diagnosis and treatment (Hanahan and Weinberg, 2000). Cancer is also a disease with significant mortality; as many as seven million deaths worldwide were attributed to this disease in 2001 alone (Danaei *et al*., 2005).

A large percentage of people will develop some form of cancer over their lifetime and, although cancer can strike any person at any age, most cancers develop in the elderly. It is estimated that 40% of women and 45% of men in Canada will develop cancer in their lifetime, and one in four Canadians will die from this disease (Canadian Cancer Society's Steering Committee: Canadian Cancer Statistics, 2009). Although cancer can develop in a large number of organs, over half of cancers affecting men and women in Canada can be attributed to three types; namely, prostate cancer, lung cancer, and colorectal cancer in men, and breast cancer, lung cancer, and colorectal cancer in women. Lung cancer is the leading cause of cancer-related death in both men and women in Canada, while colorectal cancer is responsible for the second most deaths (Canadian Cancer Society's Steering Committee: Canadian Cancer Statistics, 2009). Cancer is therefore a disease with a serious impact on society.

Both genetic and behavioural risks can contribute to the development of cancer, which occurs when cells gain a growth advantage through the mutation of different genes. Individuals may be genetically predisposed to developing cancer if they have inherited germline mutations of particular genes. For example, mutations in Breast Cancer (BRCA) 1 and BRCA2 genes are inherited in a dominant fashion in hereditary breast and ovarian cancers (Ford *et al*., 1998), while mutation in adenomatous polyposis coli (APC) is observed in one form of hereditary colon cancer (Nakamura *et al*., 1992). The presence of such mutations results in an increased risk of developing cancer in affected individuals. A large number of cancers may also develop as a result of environmental and behavioural risks. It is estimated that modifiable behaviours contributed to 35% of all cancer deaths in 2001, with the major behaviours being smoking, alcohol use, a low intake of fruits and vegetables, and being overweight or obese (Danaei *et al*., 2005). The most obvious example of the impact of behaviour on cancer incidence is likely the

link between smoking and cancer; it is estimated that 21% of cancer deaths may be attributed to smoking worldwide (Danaei *et al*., 2005). Some cancers can also develop following infection with particular viruses or bacteria. For example, the expression of human papilloma virus viral proteins can cause transformation leading to cervical cancer (Syrjänen and Syrjänen, 1985), while *Helicobacter pylori* infection can contribute to the development of gastric cancers (Loffeld *et al*., 1990). Behavioural, environmental, and genetic factors can therefore all contribute to cancer development.

Although cancer remains a disease with significant mortality, the large amount of research that has gone into understanding it has lead to various interventions capable of decreasing cancer incidence and mortality. For instance, the development of vaccines such as those for hepatitis B, which has been implicated in liver cancer, and human papilloma virus, which is a significant cause of cervical cancer, should lead to a decreased incidence of specific cancers. Furthermore, the implementation of programmes to increase awareness and encourage the modification of behavioural risk factors that can lead to cancer should also result in a decreased incidence of this disease in general. The development of better detection methods and increased screening should also lead to earlier detection and thus better prognoses due to earlier interventions. These advances, coupled to more effective chemotherapeutic and radiation therapies, should result in further decreases in the incidence and mortality of this disease in the future.

1.2.1. Cancer development and progression

1.2.1.1. Initial cancer cell growth

Cellular growth and death are normally tightly regulated. Cancer may develop when these processes become deregulated and there is an accumulation of genetic and chromosomal changes that result in a proliferative advantage (reviewed in Sadikovic *et al*., 2008). Both chromosomal instability, which can result in abnormalities in both the number and structure of chromosomes, and microsatellite instability, which refers to point mutations that occur as a result of defects in DNA repair, can increase the likelihood of cancer developing. However, cancer does not develop as often as may be suggested by the mutation rate; evidence suggests that more than one mutation is required within a given cell in order for tumourigenesis to occur. Not only do tumour cells generally exhibit several genetic alterations ranging from point

mutations to chromosome deletions, but lesions that represent intermediate stages in the transformation from normal cells to pre-malignant states to invasive tumours have also been identified (Foulds, 1954). Several types of cancer also occur in an age-dependent manner, suggesting that mutations must accumulate over time within a cell in order for it to undergo tumourigenesis. Indeed, it has been observed that four to seven mutations occur in the course of transformation (Renan, 1993). Therefore, although cells harbouring mutations are usually detected and undergo cell death, the cells that are able to evade this process may develop further mutations that lead to tumourigenesis.

The mutation or altered expression of three types of genes in particular can lead to transformation; namely, oncogenes, tumour suppressor genes, and stability genes (reviewed in Vogelstein and Kinzler, 2004). Any alteration in the expression or activity of oncogenic proteins or tumour suppressors, be it through mutation of the gene or altered expression levels, can lead to the development of cancer. Oncogenes code for proteins that promote cell survival and proliferation. Therefore, mutations that either lead to the constitutive expression of the gene product, such as through gene amplification, or in the activation of their protein product, such as may occur due to chromosomal translocations or the mutation of residues that regulate the activity of the gene product, can lead to the transformation of a cell (Vogelstein and Kinzler, 2004; Sadikovic *et al*., 2008). For instance, the oncogene k-*ras* is commonly mutated in cancers such that it remains in an active conformation, resulting in abnormal cell proliferation (Bos, 1989). The increased signalling that results from the higher activity of oncogenic proteins may ultimately lead to tumourigenesis.

Tumour suppressors, in contrast, normally function to prevent uncontrolled cell growth and cellular survival. Therefore, unlike oncogenes, which can lead to cancer through their activation, mutations of tumour suppressor genes that inhibit the activity or expression of their protein products can provide the cell with a selective growth advantage, resulting in transformation (Vogelstein and Kinzler, 2004). For instance, the classic example of a tumour suppressor protein, p53, has been found to be mutated in 50% of cancers (Soussi and Wiman, 2007). In colon cancer, the loss of p53 function frequently occurs through the missense mutation of one allele combined with the deletion of the other allele (Baker *et al*., 1989). Indeed, the portion of chromosome 17p that contains the p53 gene has been found to be deleted in over 75% of colon cancers (Vogelstein *et al*., 1988). As p53 normally functions to initiate

cell cycle arrest, senescence, or apoptosis in response to DNA damage or other cellular stresses, it is not surprising that the mutation of the p53 gene can lead to cellular transformation (Aylon and Oren, 2007). The loss or mutation of genes coding for p53 and other tumour suppressors is a common occurrence in tumourigenesis.

The loss of either the activity or expression of proteins involved in DNA damage repair can also contribute to cellular transformation (Vogelstein and Kinzler, 2004). Stability genes, which include mismatch repair (MMR) genes, nucleotide excision repair genes, and base-excision repair genes, code for proteins that act to repair DNA damage that occurs either through normal DNA replication, or through exposure to DNA damaging agents (reviewed in Friedberg, 2003). As the protein products of these genes are responsible for maintaining the genetic material of a cell with as few mutations as possible, there is an increased likelihood of mutations in other genes upon loss of function, including oncogenes and tumour suppressor genes (Vogelstein and Kinzler, 2004). The accumulation of these mutations can subsequently lead to transformation.

Epigenetic changes, or heritable changes in gene expression that do not affect the gene sequence itself, are also capable of conferring a growth advantage on a cell and can lead to cellular transformation. Such changes are common in cancer, and include alterations in DNA methylation, histone modification, and microRNA expression (reviewed in Sawan *et al*., 2008). For instance, in non-cancerous cells, the majority of the CpG islands associated with promoters are unmethylated, allowing for transcription of the associated genes, while hypermethylation of these regions is generally associated with gene silencing (reviewed in Sadikovic *et al*., 2008). However, cancer cells can have significant disruptions in DNA methylation and both hypermethylation of promoter regions and global hypomethylation are common (Ehrlich, 2002). This can lead to the development of cancer, as hypermethylation of the promoter regions of tumour suppressor genes results in the decreased expression of tumour suppressor proteins, while hypomethylation can lead to the increased expression of oncogenic proteins (Vogelstein and Kinzler, 2004). Alterations in the post-translational modifications of histones can also affect the expression of particular genes by altering the access of transcription factors and other proteins to their promoter regions (Sawan *et al*., 2008). Therefore, both mutations and epigenetic modifications that lead to the abnormal expression or activity of oncogenes, tumour suppressor genes, or stability genes, can provide a cell with a growth advantage that

results in transformation. Although hereditary predispositions to cancer can result if these mutations or epigenetic changes are present in the germline and are passed to offspring, they also commonly occur in somatic cells and lead to sporadic cancers.

1.2.1.2. Cancer cell characteristics

Transformed cells are phenotypically distinct from the normal cells from which they are derived (reviewed in Hanahan and Weinberg, 2000). These differences can include the ability to regulate their own growth and proliferation, insensitivity to growth inhibition signals, and an ability to evade apoptosis signals. In addition, cancer cells are also able to undergo an unlimited number of cell divisions and replicate at an elevated rate. Finally, a subset of cancer cells is able to promote angiogenesis and undergo metastasis. These characteristics will be discussed in the following section.

1.2.1.2.1. The enhanced growth of cancer cells

Normally, cells require growth signals to undergo proliferation, a dependency that acts to regulate cellular growth within a tissue. Tumour cells, however, are much less dependent on exogenous growth signals. Although paracrine signalling is likely also involved in signalling the majority of cancer cells to proliferate (reviewed in Hanahan and Weinberg, 2000), many tumour cells are able to secrete many of their own growth factors and signal in an autocrine manner. This allows cancer cells to grow in the absence of signalling molecules from the normal surrounding tissue (Joslin and Lauffenburger, 2006). Some tumour cells can also overcome the dependence upon growth factors by overexpressing growth receptors, which allows low levels of growth factors that would not normally result in the induction of signalling pathways to be detected, resulting in proliferation (Hanahan and Weinberg, 2000). Similarly, some cancer cells also express integrins that, upon binding the extracellular matrix (ECM), are capable of activating signalling pathways that result in cellular proliferation (reviewed in Lukashev and Werb, 1998; Giancotti and Ruoslahti, 1999). In addition to increased pro-growth signalling, cancer cells can also become insensitive to anti-growth signals (Hanahan and Weinberg, 2000). The ability to grow in the presence of anti-growth signals, as well as a decreased dependence upon pro-growth signals, provides cells with a proliferative advantage and contributes to cancer cell growth.

The ability to evade programmed cell death, or apoptosis, is also a hallmark of cancer cells that, along with increased rates of proliferation, contributes to their ability to expand in number (Hanahan and Weinberg, 2000). Normally, cells monitor themselves for abnormalities including DNA damage, aberrant signalling, an insufficiency of survival factors, loss of cell-cell or cell-ECM contacts, or hypoxia, and will undergo apoptosis if they are detected (reviewed in Evan and Littlewood, 1998). Once triggered, apoptosis results in a disruption of the cell membrane, the breakdown of the cytoplasmic and nuclear skeletons and degradation of the chromosomes, the loss of cytosol and, finally, the fragmentation of the nucleus (Kerr *et al*., 1972). In this way, damaged cells are prevented from further proliferating. Cancer cells, however, are able to expand even when damaged, which allows them to accumulate further mutations and form tumours.

Senescence, the state reached by a cell once it has gone through the maximum number of divisions possible and stops dividing, is a property of all normal cells. The number of divisions possible for a cell to make is determined by the length of the telomeres located at the ends of chromosomes; as DNA polymerase is unable to replicate the ends of chromosomes, 50 to 200 base pairs are lost from the telomeres following every cell division (Raynaud *et al*., 2008). Once the telomeres have been degraded, the maximum number of divisions by a cell has been reached and cells normally enter senescence, thereby ensuring that the multiplication of a given cell is limited (Hayflick, 1965). However, cancer cells are able to undergo further divisions by maintaining their telomeres, a phenomenon that is observed in most cancers (Shay and Bacchetti, 1997). This is accomplished in the majority of cancer cells by upregulating the expression of telomerase, the enzyme responsible for adding nucleotide repeats onto the ends of telomeres (Counter *et al*., 1992). Alternately, telomeric DNA may be exchanged interchromosomaly through recombination (Bryan *et al*., 1995; Bryan and Reddel, 1997). The maintenance of telomere length allows cancer cells to undergo an essentially limitless number of cell divisions, thereby allowing them to further expand in number. Therefore, the characteristics of cancer cells provide them with autonomy from environmental signals, allow them to evade apoptosis, and confer upon them limitless replicative potential, which lead to tumour growth. Once a tumour has reached a certain size, however, these abilities are no longer sufficient for its survival; the growth of larger tumours requires angiogenesis.

1.2.1.2.2. The ability of cancer cells to undergo angiogenesis

Angiogenesis, the process whereby new blood vessels are formed, is involved in physiological processes such as embryonic development, wound healing, and tissue regeneration, as well as in pathological tumour growth and metastasis. The ability to induce angiogenesis is important in tumour growth since the size of a tumour is limited by a lack of oxygen and nutrients. Initially, small tumours are able to use the existing vasculature of the affected individual in order to obtain sufficient oxygen and nutrients; however, after reaching a size of approximately 150 μm this becomes insufficient for the tumour to survive (Awwad *et al*., 1986), causing tumour cells to grow along existing blood vessels (Holash *et al*., 1999). However, for a tumour to grow larger than approximately 1 mm in diameter, the cells must acquire the ability to induce blood vessel formation (Gimbrone *et al*., 1972). As angiogenesis is normally tightly regulated by the balance of pro- and anti-angiogenic factors, cancer cells can induce this process through changes in the expression levels of these factors. For instance, increased levels of the pro-angiogenic factors vascular endothelial growth factor (VEGF) (Senger *et al*., 1986) and/or fibroblast growth factor (FGF) (Takahashi *et al*., 1992) are secreted by tumours when compared to normal tissue, while the expression of various inhibitors of angiogenesis, such as β-interferon and thrombospondin-1, may be downregulated (Volpert *et al*., 1995). Secreted pro-angiogenic factors induce the surrounding endothelial cells to move towards the source of these factors and form blood vessels that subsequently provide the tumour with oxygen and nutrients and, ultimately, allow it to grow larger.

1.2.1.2.3. The ability of cancer cells to invade and metastasize

Once a primary tumour has been established, a subset of tumour cells may move to a secondary site by either invading adjacent tissues or through the process of metastasis, whereby cancer cells spread to distant locations in the body by moving through the bloodstream, the lymphatic system, or the body cavity (reviewed in Hanahan and Weinberg, 2000; Oppenheimer, 2006; Geiger and Peeper, 2009). This process requires that cancer cells detach from the primary tumour, invade into either lymph or blood vessels, and move through these vessels to distant areas of the body. Once the cells have reached a suitable site, they must then extravasate from the vessels and into the tissue of the secondary site, and, finally, establish themselves in this site (Geiger and Peeper, 2009). Only a small number of cells from a primary

tumour will acquire this ability (Fidler and Kripke, 1977; Talmadge *et al*., 1982); cells that are able to invade or metastasize have altered expression of cell adhesion molecules, such as E-cadherin, integrins, and cell-cell adhesion molecules (CAMS), which results in a loss of adhesion that allows them to move away from the tumour (Hanahan and Weinberg, 2000). The loss of adhesion is a hallmark of cells undergoing the endothelial to mesenchymal transition (EMT), or de-differentiation, that is commonly observed in metastasis (Geiger and Peeper, 2009). Another hallmark of cells that have the ability to invade or metastasize is the enhanced secretion of proteases into the extracellular environment (Jechlinger *et al*., 2003). These proteases facilitate invasion by degrading the surrounding extracellular matrix (ECM). Proteases secreted by surrounding inflammatory and stroma cells may also contribute to invasion in this manner (Egeblad and Werb, 2002). Therefore, both the loss of adhesion and the secretion of proteins that affect the surrounding environment allow tumour cells to become more invasive, leading to metastasis.

Once cells have moved away from the primary tumour to a secondary site, they may then adhere and grow to form a tumour in this new site (Oppenheimer, 2006). The ability of tumours to form at a secondary site is dependent upon the microenvironment of the secondary organ, and, indeed, there is some selectivity in metastasis (Kaplan *et al*., 2005), with metastatic cells often forming secondary tumours in the liver, lungs, lymph nodes, bone marrow, and brain. Furthermore, primary tumours from a particular organ will often metastasize to the same secondary site within different patients. For example, breast cancer cells frequently metastasize first to the lymph nodes and then to the liver, lungs, and bone; colon cancers commonly metastasize to the liver; and lung cancers preferentially metastasize to the lymph nodes and then the brain (Tierney *et al*., 2009). Metastasis is of great clinical importance, as up to 90% of cancer deaths are caused by metastases rather than the primary tumour (Hanahan and Weinberg, 2000).

1.2.2. Colon cancer

One cancer in which the SFKs have strongly been implicated is colorectal cancer. Colorectal cancer, like other cancers, is a disease of uncontrolled cell growth that can affect both the rectum and colon. The colorectal mucosa is made up of an epithelium separated from the base of the colon by a lamina propria, which is a layer of loose connective tissue, and a thin

layer of smooth muscle known as the muscularis mucosae (reviewed in Ponz de Leon and Di Gregorio, 2001). The colon epithelium is formed by a single layer of columnar cells, through which water and ions are absorbed, and goblet cells, which synthesize, store and secrete mucin. These cells are continually sloughed off and replaced every four to six days by cells produced at the base of the colorectal crypts that form the interior of the colon (Lipkin *et al*., 1963). The colorectal crypts are also made up of a single layer of columnar epithelial cells that contain absorptive and goblet cells, as well as undifferentiated precursor cells (Ponz de Leon and Di Gregorio, 2001). As new cells are continually differentiating from these colon stem cells, it has been hypothesized that the disruption of their normal proliferation can lead to the development of colon cancer (Wang *et al*., 2006).

One of the earliest detectable abnormalities in the colon is the formation of clusters of mucosal cells with an abnormally large layer of epithelia known as aberrant crypt foci (ACF) (Roncucci *et al*., 1991). A small number of ACF will further progress to form adenomatous polyps, which are clusters of cells arising from uncontrolled crypt cell division. Although the majority of these polyps will not develop into cancer, a small number may become malignant. Indeed, it is believed that the majority of colon cancers develop from adenomatous polyps (Winawer *et al*., 1997), and the detection and removal of these polyps can prevent the formation of colon cancer (Winawer *et al*., 1993).

1.2.2.1. Epidemiology of colon cancer

There are large geographical variations in the incidence of colon cancer; China and South American countries have a relatively low incidence, while western industrialized nations have a much higher incidence (Parkin *et al*., 2005). Indeed, colorectal cancer is one of the most common cancers in the western world. Approximately half of the population of western countries will develop a colorectal tumour by the time they reach 70 years of age; however, of these, only 10% will develop malignancies (Kinzler and Vogelstein, 1996). Although the majority of pre-cancerous polyps do not develop into malignant tumours, colorectal cancer remains a disease with significant mortality. For instance, colorectal cancer is the third most common cancer in both males and females in Canada, with 22 000 new cases diagnosed and approximately 9000 deaths estimated in 2009 (Canadian Cancer Society's Steering Committee: Canadian Cancer Statistics, 2009). This corresponds to 13.5% and 12.1% of all cancers

diagnosed in men and women, respectively, while 12.4% of all cancer deaths in men and 11.8% in women can be attributed to colorectal cancer. Therefore, as in other western nations, colon cancer is a disease with serious incidence and mortality in Canada.

Although there is a genetic basis for the development of colon cancer, environmental factors play a role as well, possibly by affecting the risk of genetic mutations. Interestingly, there is an increase in the incidence of colon cancer among populations that have adopted a western diet after immigrating from areas of low incidence to areas of high incidence (King *et al*., 1985; Lee *et al*., 2007). This further supports the idea that diet plays a significant role in the development of this disease. In addition to diet, there are also a host of other risk factors associated with colon cancer. These include: age (Gloeckler Ries *et al*., 2003), inflammatory bowel disease (Ekbom *et al*., 1990), having first-degree relatives with colon cancer (Bonelli *et al*., 1988), or having a hereditary syndrome associated with colon cancer. Gender is also a factor, as men have a higher incidence of colon cancer than women (Gloeckler Ries *et al*., 2003). As with other cancers, tobacco use (Chao *et al*., 2000), alcohol consumption (Cho *et al*., 2004), and low physical activity (Colditz *et al*., 1997) have also been found to be significant risk factors for colon cancer. Cumulatively, these behavioural and environmental factors, as well as genetic risk factors, all contribute to the likelihood of an individual developing colon cancer.

1.2.2.2. The genetic basis of colon cancer

One of the most frequently mutated genes found in colon cancer is that of the tumour suppressor APC; mutations in this gene can be found in up to 70% of sporadic colon cancers (Powell *et al*., 1992; Suraweera *et al*., 2006). Indeed, mutations of the *APC* gene can be found as early as in ACF and are common in dysplastic polyps, suggesting that the mutation of this gene may be one of the initial steps in adenoma formation (Jen *et al*., 1994). Normally, APC acts as a suppressor of Wnt signalling by catalyzing the degradation of β-catenin. The most common APC mutations are nonsense mutations that truncate the APC protein amino-terminal to the β-catenin interacting domain, resulting in elevated levels of free cytosolic β-catenin. This allows the stabilized β-catenin to translocate to the nucleus where it acts along with T-cell factors to activate various transcription factors involved in cellular proliferation (Molatore and Ranzani, 2004). APC also has additional functions in regulating cellular adhesion, apoptosis, and chromosomal segregation that can further affect transformation (Fodde *et al*., 2001; Kaplan

et al., 2001; Qian *et al*., 2007). Mutation of the *APC* gene is therefore a significant event in the transformation of colon cells.

In addition to APC, there are several other genes that are also frequently mutated in colon cancer. For instance, the protein product of the oncogene k-*ras* has been found to be activated in approximately 50% of colon cancers (Vogelstein *et al*., 1988). The activation of this protein results in increased cellular proliferation through the Ras-Raf-Mitogen activated protein kinase (MAPK) pathway, leading to clonal expansion. Mutation of p53 has also been found in over 50% of colon cancers and is an important event in the transition from late adenoma to full-fledged carcinoma (Rodrigues *et al*., 1990; Kikuchi-Yanoshita *et al*., 1992). Indeed, progression from adenoma to colon cancer is a multistep process requiring the mutation of several genes, often including those encoding k-Ras and p53.

Although the majority of colorectal cancers are spontaneous, there are two genetically inherited forms: Familial Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal Cancer (HNPCC) (reviewed in Kinzler and Vogelstein, 1996; Rustgi, 2007). FAP, which is caused by a germline mutation of the tumour suppressor gene *APC*, is inherited in an autosomal, dominant fashion and is estimated to affect approximately 1 in 7000 to 1 in 13 000 people (Bisgaard *et al*., 1994; Kinzler and Vogelstein, 1996). People with this condition develop hundreds to thousands of benign adenomatous polyps, usually by the time they have reached thirty years of age. Although these polyps are in and of themselves not harmful, the large number of polyps increases the likelihood that one or more may become malignant to the point where the development of cancer is inevitable (Rustgi, 2007). The age at which colon cancer develops in people with FAP is also much earlier than in the general population, usually occurring by forty years of age.

The other form of genetically inherited colon cancer, HNPCC, is caused by germline mutations in essential components of the DNA MMR complex; over 90% of these are found in human mutS homologue 2 (hMSH2) and human mutL homologue 1 (hMLH1). MMR inactivation results in a nearly 1000-fold increase in the spontaneous mutation rate, thereby accelerating the time in which colon cancer develops to under 36 months. Over their lifetimes, people who harbour these mutations have an 80% risk of developing colon cancer (Rustgi, 2007). Mutations in MMR genes that result in microsatellite instability, such as those found in HNPCC can also be found in approximately 15% of sporadic colon cancers (Suraweera *et al*.,

2002). Therefore, although mutations in APC and/or MMR components are found in both sporadic and inherited colon cancers, genetically inherited forms of colorectal cancer result in the development of cancer at a much younger age than occurs in the general population.

1.2.2.3. Detection, staging, and treatment of colon cancer

Adenomatous polyps, the precursors to colon tumours, are largely asymptomatic and rarely cause detectable symptoms (Cappell, 2005). Although early stage colorectal cancer is also largely asymptomatic, late stage colon cancer commonly presents with symptoms such as abdominal pain, changes in bowel habits, weight loss, and blood in the stool (Falterman *et al*., 1974; Beard *et al*., 1995). Other symptoms can include nausea, malaise, anorexia, and abdominal distention, although these are less common. The symptoms experienced by a given patient also depend upon the location and size of the tumour, and whether the cancer has metastasized (Cappell, 2005). Since these symptoms often do not present until a more advanced, incurable stage, it is difficult to detect colon cancer by clinical means. Therefore, it is suggested that mass screening of the population be undertaken in order to detect colon cancer at an early stage, when it is largely curable (Winawer *et al*., 1997; Smith *et al*., 2003). As colon cancer risk is age related, with over 95% of cases occurring after fifty years of age, this screening generally begins at fifty (Harford, 2006). Screening allows colon cancer to be detected at earlier stages than would otherwise be likely (Mandel *et al*., 1999).

Screening for colon cancer can be carried out using a number of methods, although fecal occult blood testing (FOBT) and/or colonoscopy are generally used. FOBT examines stool samples for the presence of blood. However, the sensitivity and specificity of FOBT are less than ideal because not only does colon cancer cause bleeding only intermittently, but microscopic bleeding in the colon can also result from a number of other causes (Winawer *et al*., 1997). Therefore, any patient with a positive FOBT is referred for colonoscopy, which is both highly sensitive and specific (Harford, 2006). Indeed, colonoscopy is capable of detecting not only colon cancers, but also large polyps (> 1.0 cm) with high sensitivity, and even small polyps (< 0.6 cm) with moderate sensitivity (Rex *et al*., 1997). Another advantage of colonoscopy is that it is possible to remove polyps and take biopsy samples for pathological examination during the procedure (Cappell, 2005). It is suggested that individuals over the age of fifty have a colonoscopy every ten years in order to screen for colon cancer. Currently, there

are also investigations into a number of other screening methods to detect colon cancer in its early stages, such as fecal DNA and immunochemical tests, as well as the use of computed tomography (CT) colonography (reviewed in Harford, 2006). New screening methods will hopefully allow more colon cancers to be detected at an early stage.

It is important to properly stage colon cancer in order to determine a prognosis, as well as the correct course of treatment. Staging can be done preoperatively using CT scan, urinalysis, complete blood count, and by assessing liver and kidney function (Hawk *et al*., 2005). Following surgical staging, colorectal cancer can be classified into four stages using the tumour, node, metastasis (TNM) staging system. This staging system differentiates the extent of invasion, whether lymph nodes are involved, and if metastasis has occurred (Table 1.1). The stage at which colon cancers are detected also determines the course of treatment. For instance, colon cancers detected in stage I, where the tumour has invaded only the submucosa or muscularis, may be cured with surgery alone. However, once the cancer has metastasized, and is in stage IV, there is a very poor prognosis (Hawk *et al*., 2005). While surgery may still be used to treat the disease, chemotherapy with the cancer drug 5-fluoracil (5-FU) may be given as a palliative therapy when this is no longer a treatment option. This can increase survival and quality of life; however, the majority of patients will die within two years of developing metastatic colon cancer (Markowitz *et al*., 2002). Unfortunately, the majority of people with colon cancer are only diagnosed once the cancer has progressed to the later stages of the disease (Hawk *et al*., 2005). Given the high mortality of colon cancer when detected at later stages, it is particularly important to improve screening programmes for this disease.

Table 1.1. Colon cancer staging, therapy, and 5-year survival. Adapted from Hawk *et al*., 2005.

Stage	Primary Tumour	Lymph Node Involvement	Metastasis	Therapy	5-year suvival (%)
I	Tumour invades submucosa or muscularis	None	None	Surgery	96
II	Tumour invades muscularis or perirectal tissue	None	None	Surgery, adjuvant chemotherapy controversial	87
III	Any	Any	None	Surgery + adjuvant chemotherapy	55
IV	Any	Any	Any	Surgery or palliative chemotherapy	5

1.3. SRC FAMILY KINASES

1.3.1. Historical overview

Peyton Rous first noted how tumours could be induced in chickens when exposed to a "filterable agent" derived from a chicken sarcoma in 1911 (Rous, 1911). This agent was later found to be a virus, which became known as the Rous Sarcoma Virus (RSV). Support for the idea that RSV was involved in cancer came in 1955, when it was demonstrated that cells in a tumour induced by RSV infection not only survived the infection but secreted an infectious virus, suggesting that the virus was involved in maintaining the malignant state of the tumour (Rubin, 1955). Despite initial skepticism in the scientific community, the discovery that tumours can result from viral infection later resulted in the awarding of the Nobel Prize to Rous in 1966 (Martin, 2004).

The transforming gene of RSV was not identified to be viral Src (v-Src) until 1970 (Martin, 1970). The presence of such a gene within the genome of RSV was suggested by the finding that the RNA genomes of replicating, transformation competent strains of RSV were larger than those of non-transforming strains. This suggested that a gene missing from the transformation defective strains was responsible for transformation (Duesberg and Vogt, 1970). This was indeed the case, and oligonucleotide fingerprinting was used to identify a marker for the transforming gene close to the 3' end of the viral RNA (Wang *et al*., 1976). At the same time, a v-Src deletion mutant was used to generate a complimentary DNA probe specific for Src that was found to recognize not only v-Src, but also a homologous sequence in normal avian DNA (Stehelin *et al*., 1976). Furthermore, this sequence was also found to be conserved in vertebrate DNA. This normal cellular gene, or cellular Src (c-Src), was the first proto-oncogene, or cellular homologue of a retroviral transforming gene, identified. The discovery that retroviral oncogenes have a cellular origin led to the second Src-related Nobel Prize being awarded to Bishop and Varmus in 1989 (Martin, 2004). Upon sequencing the c-Src gene, it was discovered that it differs from v-Src by a carboxy-terminal deletion and a number of point mutations that would later be found to be important in regulating the activity of the Src proteins (Shalloway *et al*., 1981; Takeya and Hanafusa, 1983).

Although the v-Src gene was identified in 1970, it was not until 1977 that the v-Src protein was isolated by using the serum from rabbits infected with RSV to immunoprecipitate v-Src from transformed fibroblasts (Brugge and Erikson, 1977). It was discovered shortly after v-Src

was immunoprecipitated from cell extracts that this protein was a kinase (Collett and Erikson, 1978; Levinson *et al*., 1978). In fact, v-Src was the first identified tyrosine kinase, as prior to this discovery it was thought that only threonine and serine residues could be phosphorylated (Hunter and Sefton, 1980). Upon investigation, c-Src was also found to have kinase activity, although this was much lower than that exhibited by v-Src (Collett *et al*., 1978; Oppermann *et al*., 1979). The decreased kinase activity of c-Src correlated with the decreased transforming ability of this protein in comparison to v-Src; although the expression of v-Src causes transformation when it is expressed in cells (Erikson *et al*., 1980), c-Src was found to have much lower transforming ability in both mammalian cell lines and chicken embryo fibroblasts (Parker *et al*., 1984; Shalloway *et al*., 1984; Iba *et al*., 1984). Despite this, some cells were found to undergo phenotypic changes consistent with transformation when c-Src was expressed at high levels (Johnson *et al*., 1985). Therefore, due to its role in cancer, Src has historically been of great interest, and continues to be of interest. For the remainder of this work, c-Src will be referred to simply as Src.

1.3.2. Src family protein structure

There are eight members of the Src family of proteins expressed in mammals. These proteins share a common domain structure of six different functional regions, and range in size from 52 to 62 kDa (Figure 1.1) (reviewed in Brown and Cooper, 1996; Thomas and Brugge, 1997). The N-terminal domain of these proteins is a Src homology (SH) 4 domain of 15 to 17 residues in length, which may be post-translationally modified with fatty acids. These fatty acids are important in the localization of the SFKs within the cell (Silverman and Resh, 1992; Silverman *et al*., 1993). For instance, when myristoylated on Glycine-2, the SFKs can be found associated with cellular membranes or in the cytosol, while unmyristoylated SFKs do not bind membranes and remain unbound in the cytosol (Resh, 1994). The SH4 domains of all SFKs, with the exception of Src and Blk, also contain cysteine residues that may be reversibly palmitoylated post-translationally, which acts to stabilize the SFKs at the membrane (Koegl *et al*., 1994).

C-terminal to the SH4 domain is the unique domain (Figure 1.1), so named as it is the least conserved domain among the SFKs. These domains range in size from 50 to 80 amino acids (Engen *et al*., 2008). It is believed that the unique domain of each protein may be responsible

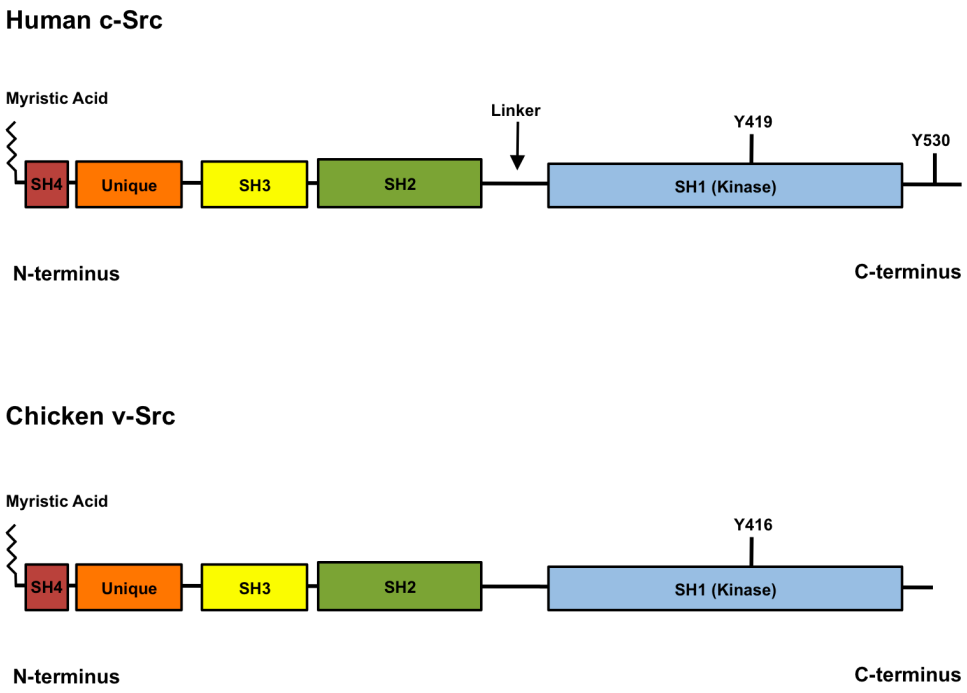


Figure 1.1. Src family kinase domain structure. The SFKs share a common domain structure, with an SH4 domain followed by a unique domain, SH3 and SH2 domains and a kinase domain. The important regulatory tyrosines (Y) of Src, Y530 and Y419, are shown; the other SFKs have corresponding regulatory tyrosines. The domain structure of v-Src is also shown. Of note is that the v-Src protein is lacking the regulatory Y530 at the c-terminal end of the protein.

for the specific interactions of each SFK with particular receptors and other protein targets. For instance, it has been shown that Lck associates with the T-cell receptors CD4 and CD8α through this domain (Veillette *et al*., 1988; Shaw *et al*., 1989). Fyn also associates with the cytoplasmic chains of the T-cell receptor through its unique domain (Timson Gauen *et al*., 1992). Therefore, it is likely that the unique domain plays a role in specifying the substrates of the different SFKs.

A non-catalytic SH3 domain of approximately 50 amino acids follows the unique domain (Figure 1.1). SH3 domains are found in many signalling molecules and are involved in both intra- and inter-molecular interactions. The SH3 domains of different proteins bind different consensus sequences; the SH3 domains of the SFKs bind proline-rich regions with the consensus sequence of P-X-X-P (Alexandropoulos *et al*., 1995), where the specificity of the

interaction is determined by the amino acids adjacent to the proline residues (Rickles *et al*., 1995). The SH3 domains of the SFKs are involved in both regulating the catalytic activity of these proteins through intramolecular interactions, and mediating intermolecular interactions with other protein substrates (Ingley, 2008).

The SH2 domain that follows the SFK SH3 domain is another protein binding domain of approximately 100 amino acids that can recognize and bind a short amino acid sequence containing a phosphotyrosine residue. Like SH3 domains, SH2 domains are also found in different protein tyrosine kinases and preferentially bind different consensus sequences (Thomas and Brugge, 1997). The specificity of individual SH2 domains is determined by the three to five amino acids following the tyrosine residue (Songyang *et al*., 1993), whereby one of the two hydrophobic binding pockets present in the SH2 domain binds the phosphotyrosine and the second interacts with the third amino acid following this residue (Eck *et al*., 1993; Waksman *et al*., 1993). The SFKs preferentially bind the sequence pY-E-E-I (Songyang *et al*., 1993). Following the SH2 domain is an SH2-kinase linker region that functions as a SH3 binding site and is involved in maintaining the SFKs in an inactive state.

The final domain of the SFKs is a 250 amino acid SH1 kinase domain, which is responsible for the catalytic activity of these proteins. SH1 kinase domains, which contain subdomains including an ATP binding pocket, are well conserved in all tyrosine kinases (Brown and Cooper, 1996). Despite the conserved nature of this domain, the consensus sequence phosphorylated by this domain varies between proteins; the SFKs preferentially phosphorylate the consensus sequence EEEIY$^{G}/_{E}$EFD (Songyang *et al*., 1995). The SFK kinase domain also contains a regulatory tyrosine residue that is necessary for the activation of the Src kinases (Smart *et al*., 1981; Brown and Cooper, 1996). A short regulatory tail of 15 to 17 amino acids that contains another phosphorylation site important in negatively regulating the kinase activity of the SFKs is also present at the carboxy terminal end of the SFKs (Cooper *et al*., 1986).

1.3.3. Src family regulation

The kinase activity of the SFKs is regulated by the phosphorylation state of key regulatory tyrosines, as well as by intramolecular interactions involving the SH3, SH2, and C-terminal domains (reviewed in Thomas and Brugge, 1997). The key tyrosine (Tyr) residues important in regulating the kinase activity of human Src are Tyr 530, which is found in the C-terminal

negative regulatory tail of the protein, and Tyr 419, which is located in the kinase domain; the other family members have corresponding residues. Normally, the majority of Src present in a cell has been phosphorylated on Tyr 530 by the Carboxy-terminal Src kinase (Csk) (Nada *et al*., 1991; Okada *et al*., 1991), or its homologue Csk homology kinase (Chk) (Davidson *et al*., 1997). When Tyr530 is phosphorylated, the SFKs are held in an inactive, closed conformation where phosphorylated Tyr 530 is bound by the SH2 domain in an intramolecular interaction (Xu *et al*., 1997), and the SH3 domain interacts with the linker region found between the SH2 and the kinase domains (Figure 1.2). The dephosphorylation of Tyr 530, however, disrupts the intramolecular associations holding the protein in the inactive conformation, which leads to its activation (Cooper and King, 1986). Indeed, the mutation or loss of Tyr 530 can lead to the constitutive activation of Src (Cartwright *et al*., 1987). Not surprisingly, v-Src, which lacks the C-terminal regulatory tail present in its cellular homologue, is also constitutively active (Takeya and Hanafusa, 1983).

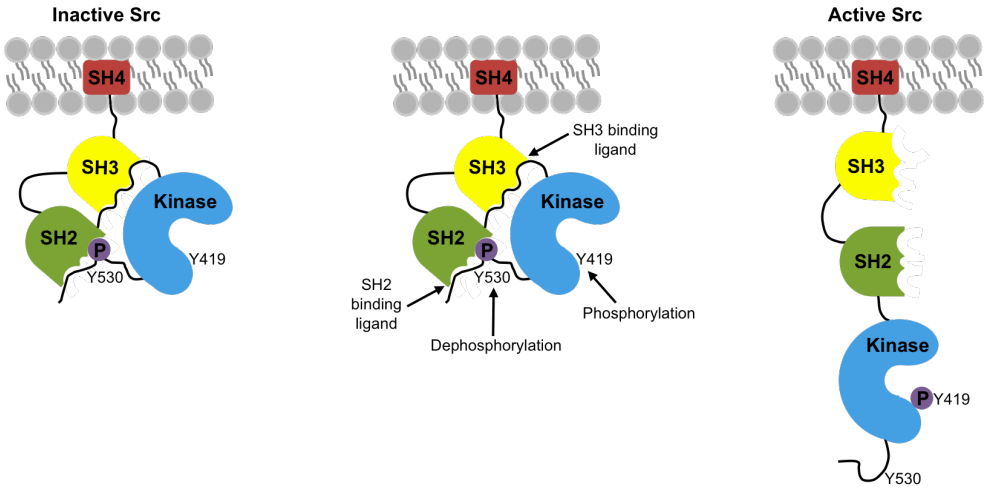


Figure 1.2. Src family kinase activation. Src is normally held in an inactive state in the cell (left panel), whereby Y530 is phosphorylated and is bound by the SH2 domain. The linker region between the SH2 domain and the kinase domain also associates with the SH3 domain when Src is in the closed conformation, thereby holding it in an inactive state. Possible mechanisms of activation are shown in the middle panel. When Y530 is de-phosphorylated and the intramolecular associations of the SH2 and SH3 domains are disrupted, Src is in an active state. The autophosphorylation of Y419 results in full activation of Src. The other SFKs are regulated in the same manner, with corresponding regulatory tyrosines. (Reproduced from Thomas and Brugge, 1997).

The interaction of the SFKs with other proteins can also disrupt the intramolecular associations that hold the SFKs in an inactive state, thereby also leading to SFK activation. For example, the SFKs bind substrates such as focal adhesion kinase (FAK) (Cobb *et al*., 1994; Thomas *et al*., 1998), $p130^{Cas}$ (Burnham *et al*., 2000), and platelet-derived growth factor receptor (PDGFR) (Kypta *et al*., 1990; Alonso *et al*., 1995) through their SH2 and SH3 domains. As these intermolecular associations have higher affinity for these domains than the intramolecular associations maintaining the SFK in an inactive conformation, the intramolecular interactions are disrupted (Bjorge *et al*., 2000a). Therefore, both the dephosphorylation of Tyr 530 and the interaction of the SFKs with other proteins can lead to the activation of the SFKs.

In addition to the dephosphorylation of Tyr 530 and the disruption of the SFK intramolecular interactions, the autophosphorylation of the other regulatory tyrosine residue, Tyr 419, is necessary for full activation of the SFKs. The phosphorylation of this residue causes it to be displaced from the substrate binding pocket, thereby allowing the SFKs to bind substrates (Xu *et al*., 1997, 1999). Therefore, the SFKs are inactive and in the closed conformation when Tyr 530 is phosphorylated, Tyr 419 is dephosphorylated, and the SH2 and SH3 domains are engaged in intramolecular interactions. They are fully active when the SH2 and SH3 domain intramolecular interactions are disrupted, Tyr 530 is dephosphorylated, and Tyr 419 is phosphorylated (Figure 1.2) (Kmiecik and Shalloway, 1987). The phosphorylation states of both Tyr 530 and Tyr 419 consequently dictate the level of SFK activity (Ingley, 2008).

As the phosphorylation states of key regulatory tyrosines are important in the regulation of SFK activity, phosphatases which dephosphorylate Tyr 419 or Tyr 530 play an important role in regulating the activity of these proteins. The dephosphorylation of Tyr 530 leading to increased SFK activity can be accomplished through the action of a number of phosphatases, including protein tyrosine phosphatase (PTP) α (Harder *et al*., 1998), PTP1B (Bjorge *et al*., 2000b), Src homology region 2 domain-containing phosphatase (SHP-1) (Somani *et al*., 1997), and SHP-2 (Peng and Cartwright, 1995; Walter *et al*., 1999), among others, whereas the dephosphorylation of Tyr 419 leads to decreased SFK activity (reviewed in Roskoski, 2005). The activity level of the SFKs therefore depends upon regulation by kinases and phosphatases, as well as on the formation and disruption of intermolecular interactions.

The involvement of the SFKs in different signalling pathways can also be regulated by the localization of these proteins within a cell. For instance, the majority of the Src protein present in fibroblasts is held in an inactive state in the perinuclear region, in either endosomes or in association with the microtubule-organizing centre (Kaplan *et al*., 1992), while constitutively active forms of Src can be found primarily in association with the cytoskeleton (Hamaguchi and Hanafusa, 1987). Indeed, inactive Src is maintained in the perinuclear region until it is activated, at which time it is translocated in endosomes to the plasma membrane (Sandilands *et al*., 2004). Upon activation, Src can therefore be found both in endosomes (de Diesbach *et al*., 2008) and at the cell surface, where it associates with peripheral membrane associated focal adhesions (Kaplan *et al*., 1994). This relocation brings Src closer to specific substrates at the plasma membrane, where Src is able to interact with integrins, growth factor receptors, and other signalling molecules that were unavailable in the perinuclear space (Sandilands *et al*., 2004). The localization of active Src into distinct cellular compartments can therefore lead to differential signalling, as different substrates become available. This may also provide one way in which specificity in signalling between the SFKs is achieved, as the subcellular localizations and trafficking of the various SFKs differs, presumably because of the differing acylations of the family members (de Diesbach *et al*., 2008; Sato *et al*., 2009). Src, which is only myristoylated, is transported to the plasma membrane by endosomes. In contrast, Lyn and Yes, which are monopalmitoylated, are transported through the secretory pathway, via the Golgi, while the dual palmitoylated Fyn is directly targeted to the plasma membrane (Kasahara *et al*., 2007; Sato *et al*., 2009). This raises the possibility that the specific functions of the SFKs may be directed, in part, through their distinct localizations within the cell by allowing interactions with the substrates located at those distinct sites (Sato *et al*., 2009). The involvement of the SFKs in signalling pathways is therefore regulated both by the phosphorylation state of the given SFK and the specific protein substrates with which it interacts.

1.3.4. Src family members

The SFKs are involved in signalling in response to interactions with a wide variety of molecules, including growth factor and immune receptors, cytokines, G-protein-coupled receptors, and adhesion molecules such as integrins (reviewed in Parsons and Parsons, 2004). Therefore, it is perhaps not surprising that this family of proteins has been implicated in a wide

variety of cellular processes, including proliferation, differentiation, adhesion, migration, cell survival, and invasion (reviewed in Brown and Cooper, 1996), and that the aberrant activation of the SFKs has been found in a wide variety of diseases, including cancer (Ishizawar and Parsons, 2004), neurodegenerative diseases (Ho *et al*., 2005), and epilepsy (Sanna *et al*., 2000).

Although the SFKs share the same basic domain structure and are often co-expressed within a given cell type, the different SFKs also have distinct features. Each of the SFKs that will be examined further in this work will therefore be discussed briefly, including their expression patterns and the phenotypes observed in knockout mice lacking a single SFK. Important features of each SFK are also summarized in Table 1.2.

Table 1.2. Src family kinase molecular weight, expression, and phenotypes of single SFK knockout mice. Adapted from Lowell and Soriano, 1996.

SFK	MW (kDa)	Expression	Phenotype of single knockout mice
Src	60	Ubiquitous; highest in brain, platelets and osteoclasts	Osteopetrosis
Yes	62	Ubiquitous, highest in brain, fibroblasts and endothelial cells	Decreased pIgA transport
Fyn	59	Ubiquitous	Abnormal hippocampal development, impaired memory, defect in thymocyte signalling
Lyn	53, 56	Brain, B-cells, myeloid cells	Autoimmunity, impaired B cell function
Lck	56	T cells, Natural Killer cells	Block in T cell development, impaired T cell receptor signalling
Hck	59, 61	Myeloid cells	None observed
Fgr	58	Myeloid cells, mature B cells	None observed
Blk	56	B cells	None observed

1.3.4.1. Src kinase

The prototype member of the Src family is Src, a 60 kDa non-receptor tyrosine kinase. Although Src is ubiquitously expressed, it is expressed at particularly high levels in platelets (Golden *et al*. 1986), neurons (Cotton and Brugge, 1983), and osteoclasts (Horne *et al*., 1992). In addition to the ubiquitously expressed isoform of Src, there are also two neuron specific isoforms that result from differential splicing of extra exons into the SH3 domain (Brugge *et al*., 1985; Martinez *et al*., 1987; Pyper and Bolen, 1990).

A great deal of insight into the cellular functions of the SFKs has come from studies of knockout mice. Despite being implicated in a variety of cellular processes, including proliferation, differentiation, and migration, the only readily observable phenotype of *src*$^{-/-}$ mice is the development of osteopetrosis, a defect in bone resorption that leads to an overgrowth of bone (Soriano *et al*., 1991). This is perhaps not surprising as Src is required for the normal differentiation and function of osteoclasts, which act to resorb bone (Lowe *et al*., 1993). More recently, *src*$^{-/-}$ mice have also been found to have a defect in lactation, implicating Src in mammary gland development as well (Watkin *et al*., 2008). These findings suggest that although Src is expressed in most cells and is involved in many cellular processes, it is not necessary for the majority of them.

1.3.4.2. Yes kinase

Like Src, the 62 kDa Yes kinase is ubiquitously expressed, although it is found in particularly high levels in fibroblasts, endothelial cells, and portions of the brain (Sudol *et al*., 1988; Lowell and Soriano, 1996). Yes and Src share the greatest sequence homology of the SFKs, with 90% homology of the kinase domain and 80% homology overall (Kitamura *et al*., 1982).

Despite its ubiquitous expression, *yes*$^{-/-}$ knockout mice do not show any readily observable phenotype (Lowell and Soriano, 1996). Indeed, the only phenotypic change in *yes*$^{-/-}$ mice is decreased transport of Immunoglobulin A (IgA) across epithelial cells, which is important in the immune response to pathogens entering the body through mucosal surfaces (Luton *et al*., 1999). However, Yes likely has at least some role in many of the cellular events regulated by Src and the other SFKs, as its expression appears to be able to compensate for the loss of other family members.

1.3.4.3. Fyn kinase

Like Src and Yes, Fyn is ubiquitously expressed. However, there are two 59 kDa isoforms of Fyn: Fyn (T) is specific to hematopoietic cells, while Fyn (B) is expressed in other cell types. These isoforms are due to alternative splicing of the Fyn gene in the seventh exon, which codes for the kinase domain (Cooke and Perlmutter, 1989). Fyn is involved in a diverse set of cellular functions through its ability to bind and phosphorylate a large number of different intracellular signalling molecules. In particular, Fyn has been implicated in cellular adhesion, T-cell signalling, and in brain functions (Resh, 1998). Evidence for the involvement of Fyn in brain functions has come from the study of Fyn knockout mice, where a significant decrease in myelination has been reported (Umemori *et al*., 1994). *fyn*$^{-/-}$ knockout mice also have abnormal hippocampal development and defects in learning and memory functions, as well as defects in thymocyte signalling (Grant *et al*., 1992; Stein *et al*., 1992).

1.3.4.4. Lyn kinase

Lyn is generally considered to be expressed mainly in immune cells. In particular, Lyn is the most highly expressed SFK in B-cells (Xu *et al*., 2005). Lyn is involved in both activating and inhibiting the activation of these cells, depending on the stimulus involved, as well as the developmental state of the cell (reviewed in Scapini *et al*., 2009). Lyn is also expressed in other cell types, including myeloid cells (Rider *et al*., 1994) and in the brain (Umemori *et al*., 1992). There are two co-expressed isoforms of Lyn that result from alternative splicing within the unique domain; one of the isoforms is 53 kDa while the other is 56 kDa (Stanley *et al*., 1991; Yi *et al*., 1991).

Lyn knockout mice have impaired B-cell function and develop lethal autoimmune disease. Although the population of B-cells present in the bone marrow of *lyn*$^{-/-}$ mice is comparable to that observed in *lyn*$^{+/+}$ mice, the number of peripheral B-cells is decreased by over 50% (Hibbs *et al*., 1995; Nishizumi *et al*., 1995). B-cells from *lyn*$^{-/-}$ mice are also hyper-responsive to B-cell signalling, as Lyn is involved in downregulating this process. This is thought to result in abnormal B-cell selection, which leads to the production of self antibodies and the subsequent development of autoimmune disease (Xu *et al*., 2005).

Despite originally being thought to have expression restricted to cells of lymphoid origin, Lyn has more recently been found to be expressed in epithelial cells. For instance, Lyn is

expressed in cultured airway smooth muscle cells (Pertel *et al*., 2006), as well as in normal prostate epithelia. Indeed, Lyn is important in prostate epithelium development, and *lyn*$^{-/-}$ mice have abnormal prostate gland morphogenesis (Goldenberg-Furmanov *et al*., 2004). It is likely that Lyn has important functions in other epithelial tissues as well.

1.3.4.5. Other Src family members

The expression of the other SFKs, Lck (56 kDa), Hck (59 kDa), Fgr (58 kDa), and Blk (56 kDa), is mainly restricted to hematopoietic cells, although Hck and Fgr are also expressed at high levels in osteoclasts in mice (reviewed in Lowell and Soriano, 1996). Hck and Fgr are, however, expressed primarily in neutrophils, monocytes and macrophages, while Fgr is also expressed in mature B-cells (Brown and Cooper, 1996). These two proteins, as well as Lyn, have also been found to be involved in a variety of intracellular signalling pathways in myeloid cells, including cytokine receptor signalling (Corey *et al*., 1993; Corey *et al*., 1994; Anderson and Jorgensen, 1995), fc fragment of IgG receptor (FcγR) signalling (Hamada *et al*., 1993; Wang *et al*., 1994), and the lipopolysaccharide response (Stefanova *et al*., 1993). Of the two other SFKs, Lck is expressed mainly in T-cells and natural killer cells, while Blk is mainly expressed in B-cells. Interestingly, while *lck*$^{-/-}$ mice exhibit a block in T-cell development, as well as impaired T-cell receptor signalling (Molina *et al*., 1992; Wen *et al*., 1995), *hck*$^{-/-}$, *fgr*$^{-/-}$, and *blk*$^{-/-}$ mice do not show any observable phenotypic changes (Lowell and Soriano, 1996). The non-ubiquitous SFKs are, however, important in signalling in various immune cells.

1.3.5. Src family redundancy

As the SFKs are often co-expressed, the possibility exists that they have redundant or compensatory functions. Some of the best evidence to support this has come from studies involving Src family knockout mice, which suggest that there is indeed a great deal of functional overlap between the SFKs (Lowell and Soriano, 1996). As in humans, the SFKs Src, Yes, Fyn, Lyn, Lck, Hck, Fgr, and Blk are expressed in mice. While Src, Yes, Fyn, and Lyn are expressed in a wide variety of tissues, with particularly high levels of these proteins found in neurons and hematopoietic cells, the expression of the other four SFKs is more restricted to hematopoietic cells (Lowell and Soriano, 1996). Surprisingly, as discussed in the previous section, all of the single Src family knockout mice are viable and exhibit only

relatively mild phenotypes. Given the wide expression of these proteins and their involvement in both development and a number of other cellular processes, this suggests that there may be functional overlap between the SFKs.

In addition to knockout mice lacking single SFKs, mice in which different combinations of the SFKs have been knocked out have been developed. When contrasted to the phenotypic changes observed in single knockout mice, it is obvious that the defects in these mice are much more severe (reviewed in Lowell and Soriano, 1996). Although *src*$^{-/-}$, *yes*$^{-/-}$ and *fyn*$^{-/-}$ mice are all viable, *src*$^{-/-}$*yes*$^{-/-}$ and *src*$^{-/-}$*fyn*$^{-/-}$ double knockout mice die perinatally (Stein *et al*., 1994). Furthermore, while approximately one third of *fyn*$^{-/-}$*yes*$^{-/-}$ mice are viable, they exhibit much more severe defects than their corresponding single knockouts; these mice have enlarged spleens and severe renal disease due to glomerulosclerosis (Stein *et al*., 1994). These findings suggest that there is significant functional overlap between the SFKs, and that they are able to compensate for the loss of activity of other family members. In mice that have had multiple SFKs knocked out, the SFKs that could normally compensate for this loss are also absent, leading to a more severe phenotype (Lowell and Soriano, 1996). Redundancy in specific signalling pathways in which the SFKs are involved has also been found, where the same stimulus results in the activation of more than one SFK. For example, Src, Yes, and Fyn have all been found to be activated following the stimulation of PDGFR (Kypta *et al*., 1990). SFK redundancy is also complicated by the fact that a single SFK may be activated by multiple different stimuli, and that, in addition to any redundant functions, the SFKs may have unique functions in different cell types (Lowell and Soriano, 1996).

Further evidence suggesting that the SFKs are able to compensate for the loss of one of the family members has come from examining the expression of the other SFKs in the different knockout mice. For instance, the level and activity of Src is increased in neurons from *fyn*$^{-/-}$ mice, suggesting that Src may be able to compensate for the loss of Fyn by phosphorylating various substrates that would normally be phosphorylated by Fyn (Stein *et al*., 1994). Macrophages from *hck*$^{-/-}$ mice also have increased Lyn activity (Lowell *et al*., 1994), while the level of Hck is increased in osteoclasts from *src*$^{-/-}$ mice. Interestingly, *src*$^{-/-}$*hck*$^{-/-}$ double knockout mice exhibit a much more severe osteopetrosis than do the single knockout mice, strongly suggesting that Hck is able to compensate for the lack of Src in *src*$^{-/-}$ osteoclasts

(Lowell *et al*., 1996). It is therefore likely that the SFKs share many redundant functions in several tissues, in addition to any individual functions that they may have.

1.4. SRC FAMILY KINASES IN CANCER

1.4.1. Src family kinase involvement in cancer

Src has been found to be overexpressed and/or activated in a number of cancers, including those of the colon, breast, pancreas, lung, ovaries, stomach and liver, as well as chronic myelogenous leukemia (CML) and multiple myeloma (Budde *et al*., 1994; Muthuswamy *et al*., 1994; Verbeek *et al*., 1996; Lutz *et al*., 1998; Masaki *et al*., 1999; Masaki *et al*., 2000, Masaki *et al*., 2003; Wiener *et al*., 2003). In addition, many of the other SFKs have also been implicated in various human epithelial and lymphoid cancers. Not surprisingly, the more ubiquitously expressed SFKs, such as Src, Yes and Fyn, are involved in both solid and hematopoetic cancers, while those with expression restricted to cells of lymphoid origin are involved mainly in leukemias and lymphomas. The involvement of the different SFKs in selected human cancers, as well as evidence implicating these proteins in changes in tumour cell growth and survival, cellular adhesion, migration, angiogenesis, and metastasis (reviewed in Irby and Yeatman, 2000), will be discussed in the following section.

1.4.1.1. Src family kinase expression and activity in colon cancer

Although Src has been implicated in a variety of human cancers, its involvement in colon cancer progression is likely the most extensively characterized. Src activation has been reported in up to 80% of colon tumours, with the level of activation correlating with colon tumour progression (Talamonti *et al*., 1993). In other words, although Src kinase activity is increased early on in the development of colon cancer, it is even higher in later stages of cancer progression. Indeed, even in the normal colon epithelia, the actively dividing crypt cells have higher levels of Src activity than the more differentiated cells (Cartwright *et al*., 1993). In addition, the level of Src activity is further elevated in the colonic polyps that are precursors to colon carcinoma; the polyps with less potential for malignancy have lower activity than the large benign polyps with the greatest potential to develop into a malignancy (those larger than 2 cm with villous structure and severe dysplasia), while malignant polyps have the highest activity (Cartwright *et al*., 1990). This suggests that Src activation may be an early event in the

transformation from normal colon cells to carcinoma cells. Further to this, Src protein levels and kinase activity have been found to be higher in primary tumours when compared to the polyps (Talamonti *et al*., 1993). Src expression and activity in liver metastases from primary colon tumours have also been examined, as the liver is the most frequent site of metastasis from colon cancer, and not only are significant increases in both Src activity and protein levels observed when compared to the normal colonic mucosa, but the liver metastases have significantly higher Src activity than the primary tumours (Talamonti *et al*., 1993). Taken together, these studies demonstrate that Src activity increases as colon cancer progresses from pre-malignant polyps to liver metastases.

The increased level of Src kinase activity in the later stages of colon cancer cannot be accounted for by the increased level of Src protein expressed in these stages alone, suggesting that Src activity may be regulated by other mechanisms than simply protein level (Talamonti *et al*., 1993). One explanation for this could be the presence of an activating mutation, and, in 1999, it was reported that a mutation resulting in the truncation of Src at codon 531 was present in 12% of the advanced human colon cancers tested that was absent in both normal tissue samples and in the early stage, primary colon cancers investigated (Irby *et al*., 1999). However, this finding has been controversial, as no other group has been able to repeat these results (Daigo *et al*., 1999; Nilbert and Fernebro *et al*., 2000) and, to date, there has been no other activating Src mutation identified. Mutations, therefore, are not likely an important mechanism of Src activation in colon cancer cells. Regardless, Src kinase activity in primary colorectal cancer has been found to correlate to poor patient prognosis (Aligayer *et al*., 2002), and increased Src expression and activity in primary colon tumours appears to be independent of age and sex of the patient, stage, or tumour location (Talamonti *et al*., 1993).

Other SFKs have also been found to be highly expressed and activated in a subset of colon cancer cell lines and primary colon cancers when compared to non-cancerous colon cells (Park *et al*., 1993). Like Src, Yes activity has been found to increase with colon cancer progression (Park *et al*., 1993; Peña *et al*., 1995). Lck is also expressed in a small subset of colon cancer cell lines. Although Lck expression is regulated by both a proximal and distal promoter in hematopoietic cells (Takadera *et al*., 1989), it is expressed exclusively from the proximal promoter in colon cancer cells (Nakamura *et al*., 1996; McCracken *et al*., 1997). Finally, although Lyn has traditionally been thought of as being expressed primarily in B-cells, it has

been implicated in colon cancer cell migration in response to cluster of differentiation 44 (CD44) (Subramaniam *et al*., 2007), as well as in the development of chemoresistance through the induction of the phosphoinositide 3'-kinase (PI3K)/Akt cell survival pathway (Bates *et al*., 2001). Therefore, Lyn may also play a role in the development of colon cancer through its ability to suppress apoptosis. These findings suggest that several of the other SFKs, in addition to Src, have a role in colon cancer.

1.4.1.2. Src family kinase expression and activity in other cancers

Src is also overexpressed and active in many other human cancers in addition to colon cancer, including breast cancer and lung cancer. For instance, Src activity is higher in breast cancer tissue than in normal breast tissue (Finn, 2008), while Src expression and activity are also increased in malignant lung tissue. One study found Src levels to be elevated in 60% of the lung tumour biopsies investigated (Mazurenko *et al*., 1992). More recently, Src expression and activity have been found to be increased in adenocarcinomas of the lung when compared to the surrounding lung tissue, with the highest Src activity observed in larger tumours (Masaki *et al*., 2003). A further study also found Src activation to be significant in squamous cell lung tumours (Zhang *et al*., 2007). Therefore, Src likely plays a role in breast and lung cancers, as well as in colon cancer.

Src expression and activity have also been found to be increased in a number of other cancers. For instance, despite a lack of Src expression in normal ovarian epithelial cells, Src is overexpressed and activated in late-stage ovarian tumours, as well as in a number of human ovarian cancer cell lines (Wiener *et al*., 2003). Similarly, Src, as well as Lyn and Fgr, are expressed at high levels in prostate cancer, although these SFKs have also been found to be expressed at lower levels in primary cell cultures derived from the prostate (Goldenberg-Furmanov *et al*., 2004; Nam *et al*., 2005). The level of Src protein expressed in neuroblastoma cell lines is also higher than in primary cultures derived from normal central nervous system tissues, while Src expression in neuroendocrine tumours has been found to increase with the differentiation state of the tumour (O'Shaughnessy *et al*., 1987). Finally, Src has been found to be overexpressed and activated in pancreatic carcinoma cell lines (Lutz *et al*., 1998). The high levels of Src activity in these cancers suggest that this protein is involved in the progression of a large number of different cancers.

The other SFKs are also overexpressed and activated in different cancers. For instance, although Src activity is not increased in melanoma cells, Yes has been found to have higher activity in both human melanomas and malignant melanoma cell lines than in melanocytes (Loganzo *et al*., 1993). The SFKs have also been found to be active in glioblastomas, and increases in Lyn activity, in particular, have been reported. In one study, it was found that although Lyn activity accounted for only approximately 30% of the total SFK activity in the normal brain, neoplastic brain, and anaplastic astrocytoma samples examined, over 90% of the Src kinase activity in glioblastoma cells was due to Lyn (Stettner *et al*., 2005). In a more recent study, Src, Fyn, Yes, Hck, and Blk were also found to be overexpressed in glioblastomas, while Lyn and Hck expression was found to be restricted mainly to mesenchymal glioblastomas (Lu *et al*., 2009). Such findings suggest that the various SFKs are involved in a number of different human cancers.

1.4.2. Effects of Src family kinase expression on tumour growth

Evidence implicating the SFKs in a number of cancer cell phenotypes has come from examining tumour specimens, as well as from tissue culture studies and *in vivo* models of various cancers. Many of these studies have either varied the expression level of Src and/or the other SFKs, or have inhibited their kinase activity. For instance, HT29 colon cancer cells expressing antisense against Src have decreased proliferation when compared to the parental cell line (Staley *et al*., 1997). The breast cancer cell line MCF7 also has decreased proliferation, as well as altered migration, attachment, and cell spreading when Src activity is decreased either through the expression of a dominant-negative form of Src, or the presence of small interfering RNAs (siRNAs) targeting Src (González *et al*., 2006). Lyn has also been implicated in the proliferation of hematopoietic cancer cells. For instance, Lyn is constitutively active in leukemia cells, and the proliferation of these cells has been shown to be inhibited when Lyn expression is reduced using antisense RNA (Roginskaya *et al*., 1999). Lyn siRNA has also been found to cause breakpoint cluster region (BCR)- c-Abl (Abl) oncogene precursor blast cells to have decreased proliferation and to undergo apoptosis, while normal hematopoietic cells are unaffected (Ptasznik *et al*., 2004), suggesting that Lyn may be a valid target for therapy in chronic myelogenous leukemia (CML). In contrast, no changes in proliferation, adhesion, or wound migration were observed in SKOv-3 ovarian cancer cells

when Src levels were decreased using antisense, although the anchorage-independent growth of these cells was significantly reduced (Wiener *et al*., 1999). A decrease in anchorage-independent growth, along with an increase in apoptosis, was also observed in breast and colon cancer cell lines in which Src levels were decreased using siRNA (Zheng *et al*., 2008). Interestingly, siRNAs targeting either Fyn or Yes had no effect on the anchorage-independent growth of the breast cancer cells investigated in this study, indicating that Src alone may be involved in cell survival following loss of adhesion in these cell lines (Zheng *et al*, 2008). Taken together, these studies suggest that the involvement of the SFKs in cancer phenotypes such as proliferation and survival may depend on the type of cell and the SFK being investigated.

In addition to proliferation and cell survival, Src has also been found to be important in later stages of cancer progression; namely, angiogenesis, invasion, and metastasis (reviewed in Yeatman, 2004). Several studies examining the ability of cells with decreased Src kinase activity to form tumours when injected into mice have been carried out. For instance, not only were HT29 colon cancer cells with decreased Src expression due to antisense found to have decreased proliferation, but the tumours formed following the injection of these cells into nude mice also grew much more slowly than would be expected from the decrease in cell proliferation observed in tissue culture (Staley *et al*., 1997). In addition, the tumours that did form from these cells were decreased in size even after a year (Ellis *et al*., 1998), thereby suggesting that Src plays an important role in the growth of colon tumours. Similarly, when MCF7 breast cancer cells expressing a dominant-negative form of Src were injected into mice, it was observed that although there was no significant change in the number of tumours, they grew more slowly due to both a decrease in proliferation and an increase in apoptosis of the tumour cells (González *et al*., 2006). Decreased tumour size and metastasis were also observed in an orthotopic mouse model of pancreatic cancer created by injecting mice with L3.6 pl pancreatic cells transfected with an siRNA targeting Src, although neither effects on proliferation nor a decrease in tumour number were observed (Trevino *et al*., 2006). Similarly, tumour size and metastasis were again observed to be reduced when mice bearing tumours formed from L3.6. pl cells were treated with the Src/Abl selective inhibitor BMS-354825 (Trevino *et al*., 2006). Pancreatic cancer cells either treated with the clinically relevant SFK inhibitor dasatinib or with an siRNA-mediated decrease in the expression of Src have also been

shown to have decreased proliferation, migration, and invasion, while dasatinib treatment also inhibited the growth of pancreatic tumours *in vivo* (Nagaraj *et al*., 2010). Finally, when ovarian cancer cells expressing antisense targeting Src were injected into nude mice there was a dramatic reduction in both vascularization and tumour development (Wiener *et al*., 1999). Taken together, these studies provide strong evidence that although cells with decreased SFK activity are able to form tumours in *in vivo* mouse models of cancer, the growth of these tumours is significantly impeded, suggesting that the SFKs are intimately involved in later stages of cancer progression.

1.4.3. Src family kinase involvement in cellular processes in cancer cells

As the SFKs are involved in a variety of cellular processes, it is perhaps not surprising that the overexpression of these proteins can lead to the aberrant signalling of multiple pathways within a cell. However, rather than through activation by mutation, the SFKs appear to be involved in signalling in cancer cells mainly through their association with other proteins that are themselves abnormally activated or expressed. Indeed, the SFKs can be activated downstream of various receptor tyrosine kinases (RTKs), G-protein-coupled receptors, cytokines, steroid receptors, CAMS, and integrins (reviewed in Ishizawar and Parsons, 2004). Therefore, if any of these proteins are more highly expressed or activated in cancer cells than in non-cancerous cells it can lead to the abnormal activation of the SFKs and the subsequent initiation of various signalling pathways involved in cancer development and progression. The involvement of the SFKs in proliferation, survival, adhesion, migration, angiogenesis, and metastasis will be discussed in the following section.

1.4.3.1. Src family kinase involvement in proliferation and cell cycle progression

The SFKs have been implicated in the proliferation of both cancer and normal cells in response to a number of stimuli, including the stimulation of both integrins and growth factor receptors. Indeed, some growth factors, such as platelet derived growth factor (PDGF), colony stimulating factor type 1 (CSF-1), fibroblast growth factor (FGF), and epidermal growth factor (EGF), require SFKs in order to induce fibroblasts to undergo DNA synthesis (Roche *et al*., 1995b). The SFKs are not required for such a response to lysophophatidic acid (LPA) and bombesin, however (Roche *et al*., 1995b; Kilkenny *et al*., 2003). When growth factor receptors

are stimulated by growth factors, the SFKs may associate with the activated receptors through their SH2 domains, leading to their activation and association with focal adhesion kinase (FAK) (Bromann *et al*., 2004; Yeatman, 2004). The non-receptor tyrosine kinase FAK is a particularly important SFK substrate, as it, along with the SFKs, is involved in regulating cell cycle progression, as well as survival, adhesion, and migration (Schaller, 2001). Integrin stimulation can also contribute to cellular proliferation by activating FAK. Following an association with integrins, the regulatory Tyr 397 of FAK is autophosphorylated, which creates a binding site for the SH2 domain of the SFKs (Schaller *et al*., 1994; Eide *et al*., 1995). This binding results in the activation of the SFK, which enhances FAK activity and provides docking sites for other signalling proteins by phosphorylating FAK at several additional tyrosine residues (Chaudhary *et al*., 2002; Mitra *et al*., 2005). Src-FAK signalling, whether in response to growth factors or integrin stimulation, subsequently results in the induction of a variety of interconnected signalling pathways that have consequences for cellular proliferation, survival, adhesion, migration, and invasion (Ishizawar and Parsons, 2004). Of these, the activation of the Ras-extracellular signal-regulated kinase (ERK)-MAPK signalling pathway in response to Src-FAK signalling is a major way in which SFK activation leads to cellular proliferation (Figure 1.3) (Giancotti and Ruoslahti, 1999). Alternately, this pathway may be

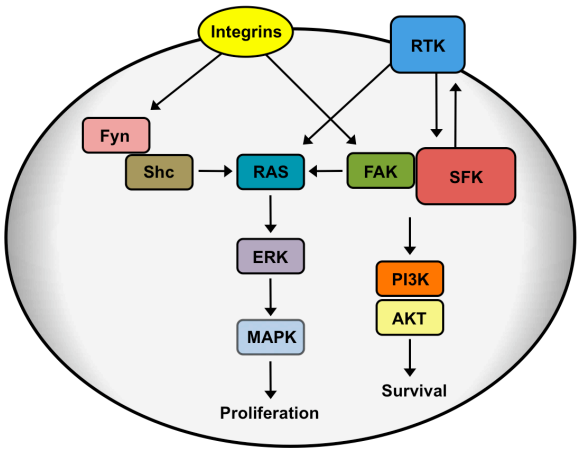


Figure 1.3. Src family kinase signalling pathways involved in proliferation and survival. Upon stimulation of integrins or RTKs, SFKs and FAK associate, leading to their activation. SFK-FAK activation can result in the activation of both the Ras-ERK-MAPK pathway, which is involved in cellular proliferation, and the PI3K-AKT pathway, which is involved in cellular survival. Alternately, a subset of integrins is able to activate Fyn directly, leading to the activation of the adaptor protein Shc and the induction of the Ras-ERK-MAPK pathway. (Modified from Kim *et al*., 2009b).

activated independently of Src-FAK signalling by a subset of integrins that are able to bind the SH3 domain of Fyn directly (Wary *et al*., 1998). Fyn subsequently recruits and activates Shc, which acts as a protein adapter leading to the activation of Ras. The activation of the Ras-ERK-MAPK signalling pathway, leading to cellular proliferation, is therefore a major role of the SFKs in cancer cells.

The SFKs also control proliferation and progression through the cell cycle by modulating the activity of proteins such as p27 and various cyclins at different points in the cell cycle. p27 normally regulates cellular proliferation by inhibiting the kinase activity of cyclin-Cdk2, which is a complex that acts to prevent entry into the S phase of the cell cycle (Chu *et al*., 2007). The phosphorylation of p27 by Src results in accelerated p27 proteolysis, which allows a progression to S phase and DNA synthesis (Chu *et al*., 2007). Lyn and Yes have also been found to phosphorylate p27, suggesting that other SFKs may also be involved in regulating cell cycle progression in this manner (Chu *et al*., 2007). In addition to downregulating p27 expression, Src also causes the upregulation of a number of cyclins, which are proteins involved in controlling cell cycle progression. For instance, the transformation of cells with v-Src results in the upregulation of cyclins D1, E, and A, which causes cells to leave quiescence and enter S phase (Riley *et al*., 2001). Therefore, in addition to activating the Ras-ERK-MAPK pathway, the SFKs are involved in regulating cell cycle progression by affecting the expression of various other proteins involved in this process.

Yes and Fyn, in addition to Src, are also activated at mitosis and have been shown to be involved in the transition from G2 to M phase in fibroblasts. Interestingly, there appears to be functional redundancy between these family members, as although treatment of fibroblasts with an antibody that neutralizes Src alone did not cause cell cycle arrest, one that neutralizes Src, Yes, and Fyn was found to inhibit cell division (Roche *et al*., 1995a). This suggests that the various SFKs can compensate for the loss of function of another family member, at least in proliferation. However, Src and Yes are likely regulated differently during mitosis, as the specific activity of Src has been found to increase two to threefold during mitosis in HT29 colon cancer cells, while Yes protein levels and activity decrease during this period as a consequence of both increased degradation and decreased synthesis (Park *et al*., 1995). Nevertheless, multiple SFKs are likely involved in signalling leading to cellular proliferation.

1.4.3.2. Src family kinase involvement in cell survival

The ability of cells to survive signals that would otherwise cause their death is another important property of cancer cells. Normally, the viability of a cell is monitored intracellularly, and if abnormalities such as DNA damage, an insufficiency of survival factors, aberrant signalling caused by oncogenes, or hypoxia are detected, the apoptosis pathway is activated (Evan and Littlewood, 1998). In addition, apoptosis may be triggered if survival signals normally present due to cell-extracellular matrix and cell-cell adhesion are absent (Giancotti and Ruoslahti, 1999). Resistance to anoikis, or detachment-induced apoptosis, is a property of many epithelial cancers that has implications in tumour cell survival and metastasis.

The involvement of the SFKs in anoikis was initially suggested by the finding that the transformation of epithelial cells with v-Src could prevent them from undergoing this process (Frisch and Francis, 1994). A number of more recent studies in various cell lines have also implicated the SFKs in anoikis. For instance, both Src and Fyn have transiently increased kinase activity in intestinal epithelial cells following detachment (Loza-Coll *et al*., 2005). Src activity is also higher in lung tumour cells that are resistant to anoikis than in those that are susceptible (Wei *et al*., 2004). Furthermore, it has also been observed that colon cancer cell lines with naturally lower levels of Src kinase activity have an increased susceptibility to anoikis when compared to cells with higher levels of Src kinase activity (Windham *et al*., 2002). The finding that colon cancer cells with naturally higher levels of Src kinase activity could be made more susceptible to anoikis by either decreasing Src expression or treating the cells with a SFK inhibitor provides more direct evidence for the involvement of the SFKs in anoikis resistance (Windham *et al*., 2002). Similarly, the treatment of osteosarcoma cells with a SFK inhibitor has also been shown to restore the sensitivity of these cells to anoikis, further confirming the involvement of the SFKs in this process (Diaz-Montero *et al*., 2006). Taken together, these studies strongly suggest that the increased SFK activity found in cancer cells contributes to anoikis resistance and the survival of these cells.

One way in which the SFKs contribute to cell survival is through the activation of the PI3K-Akt pathway. This pathway is activated in response to Src-FAK signalling following integrin or RTK stimulation, and results in the suppression of apoptosis, leading to cell survival (Figure 1.3) (Siesser and Hanks, 2006). Evidence for the involvement of the PI3K-Akt pathway in anoikis resistance following Src activation has come from studies where the treatment of cells

with SFK inhibitors prevented the activation of this pathway in response to cell detachment (Loza-Coll *et al*., 2005; Diaz-Montero *et al*., 2006). It has also been found that inhibiting PI3K results in cells regaining sensitivity to anoikis (Diaz-Montero *et al*., 2006), further supporting the involvement of this pathway in anoikis resistance. In addition to the activation of the PI3K pathway, the SFKs Fyn and Yes may also play a role in anoikis through the phosphorylation of the 135 kDa CUB-domain-containing protein 1 (CDCP1); the phosphorylation of this protein is associated with an increased resistance to anoikis (Uekita *et al*., 2007). At least some of the SFKs therefore have a role in cancer progression through their ability to confer resistance to anoikis upon cells that have lost attachment. As this is necessary for cancer cells to invade and metastasize, this is yet another way in which the SFKs contribute to cancer progression.

1.4.3.3. Src family kinase involvement in adhesion

The SFKs, in particular Src and Fyn, are involved in cellular adhesion to both the extracellular matrix (ECM) and to other cells. The actin cytoskeleton of cells is linked to the ECM through the integrin family of transmembrane receptors in points of interaction known as focal adhesions (Danen and Yamada, 2001). Integrins do not themselves possess kinase activity; however, signals may be transmitted into cells in response to interactions with the ECM through the activation of integrin-associated proteins present within the focal adhesions, such as the SFKs. As integrins can interact with a variety of proteins, as well as the actin cytoskeleton, their stimulation can initiate a number of signalling events that can regulate cellular proliferation, survival, adhesion, and migration in both normal and cancerous cells (reviewed in Gabarra-Niecko *et al*., 2003). Important components of focal adhesions, which can be composed of over 50 different proteins, include the SFKs and the non-receptor tyrosine kinase FAK (Zamir and Geiger, 2001). Upon integrin stimulation, FAK is recruited to the sites of integrin clustering by integrin-associated proteins such as paxillin, and a FAK-Src signalling complex is formed, as discussed previously. Src is subsequently able to phosphorylate other proteins involved in integrin-mediated signalling and cytoskeletal events, including FAK itself, $p130^{Cas}$, paxillin, and cortactin (Vuori *et al*., 1996; Klinghoffer *et al*., 1999; Volberg *et al*., 2001), which can lead to changes in the actin cytoskeleton that result in the disruption of the focal adhesions. Indeed, Src has been found to be integral to the phosphorylation of other proteins following integrin stimulation as few proteins are phosphorylated in response to this

stimulation in fibroblasts lacking Src, Yes, and Fyn (Klinghoffer *et al*., 1999). Src inhibition has also been found to correlate to decreased phosphorylation of Src substrates, such as FAK and $p130^{Cas}$, in a variety of cell lines (Shin *et al*., 2004; Brábek *et al*., 2005; Shor *et al*., 2007; Park *et al*., 2008; Vultur *et al*., 2008). The high SFK activity observed in cancer cells therefore has implications in the adhesion of these cells to the ECM.

The SFKs are also involved in cell-cell adhesion. The points of contact between two cells are known as adherens junctions, one of the major components of which are transmembrane proteins known as cadherins. The extracellular domain of these proteins can adhere to cadherins on other cells, while the cytoplasmic domain binds to β-catenin, thereby linking cadherins to the actin cytoskeleton. The SFKs are also components of adherens junctions; Src, Yes, and Lyn have been identified in the adherens junctions of rat hepatocytes, while Src and Yes are also enriched in those of cultured hepatocytes, primary cultured keratinocytes, and kidney epithelial cells (Tsukita *et al*., 1991). Activated SFKs are able to induce the endocytosis of E-cadherin, leading to the disruption of cadherin/catenin complexes, and, subsequently, to decreased adhesion and enhanced migratory and invasive capacity of the cells (Figure 1.4) (Avizienyte *et al*., 2002; Irby and Yeatman, 2002). The phosphorylation of p120-catenin, which is a cytoplasmic protein involved in adhesion through its association with the cadherins, by Src can also lead to cadherin turnover, further implicating these proteins in adhesion (Reynolds *et al*., 1989, 1994; Reynolds and Roczniak-Ferguson, 2004) (Figure 1.4) (Shibamoto *et al*., 1995). SFK involvement in this loss of cell-cell adhesion is supported by the finding that treatment of colon cancer cell lines with a SFK inhibitor results in increased cell-cell adhesion (Calcagno *et al*., 2005). Furthermore, both the expression of a dominant-negative Src protein and treatment with a SFK inhibitor have been shown to restore adhesion in colon cancer cell lines expressing an active form of Src (Irby and Yeatman, 2002). The treatment of keratinocytes with a SFK inhibitor, or the overexpression of a dominant-negative Src, were also able to induce and stabilize cell-cell contacts, thereby suggesting that the catalytic activity of at least one of the SFKs is required for the disassembly of adherens junctions (Owens *et al*., 2000). The finding that keratinocytes from *fyn*$^{-/-}$ mice have defects in the formation of cell-cell adherens junctions *in vitro* also demonstrates the involvement of SFKs in cell-cell adhesion. Interestingly, keratinocytes from neither *src*$^{-/-}$ nor *yes*$^{-/-}$ mice exhibit this defect, suggesting that Fyn in particular is involved cell-cell adhesion, at least in these cells (Calautti *et al*., 1998).

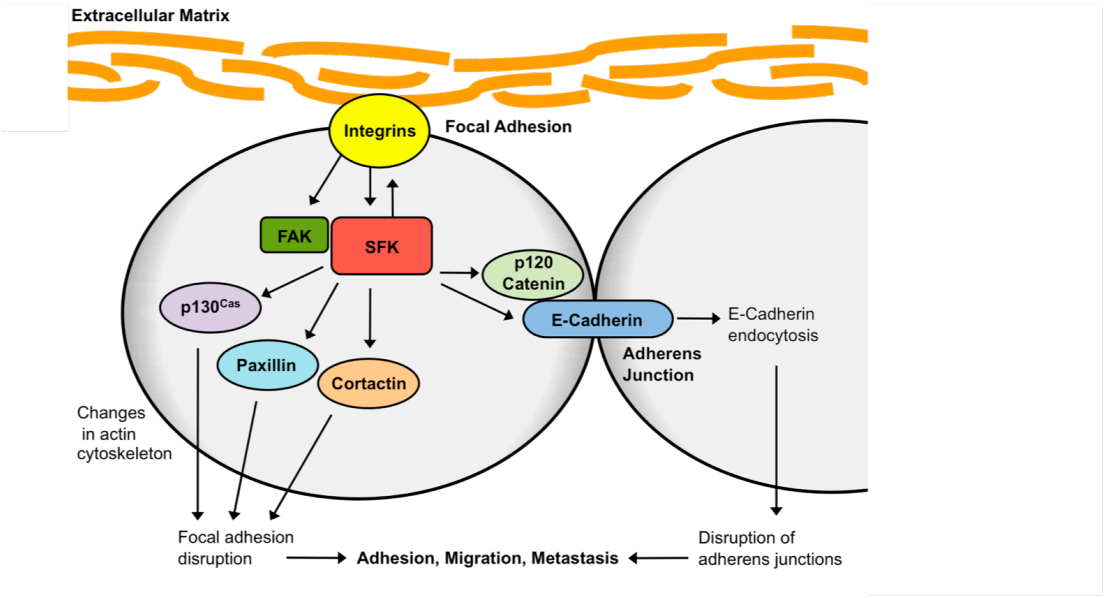


Figure 1.4. Src family kinase signalling pathways involved in adhesion, migration, and metastasis. Src and FAK become activated in response to integrin stimulation, leading to the phosphorylation of several other components of focal adhesions, including $p130^{Cas}$, paxillin, and cortactin. This can lead to focal adhesion turnover, resulting in increased cellular migration and metastasis. Active SFKs are also able to phosphorylate E-cadherin and p120 catenin, which leads to the disruption of the cadherin/catenin complex through the endocytosis of E-cadherin, the subsequent loss of cell-cell adhesion, and enhanced migration and invasion. (Modified from Yeatman, 2004 and Kim *et al*., 2009b).

Therefore, the SFKs have been implicated in both the loss of cell-cell contacts through the disruption of adherens junctions, as well as in cellular adhesion to the ECM through their role in focal adhesion disassembly, and are, in consequence, intimately involved in cellular adhesion.

1.4.3.4. Src family kinase involvement in migration

As SFK activity is required for both the disruption of integrin-dependent cellular adhesions (Fincham and Frame, 1998; Cary *et al*., 2002; Brunton *et al*., 2005), and cadherin-mediated cell-cell contacts (Owens *et al*., 2000), it is not surprising that the SFKs also play an important role in cellular migration, as well as in adhesion. The SFKs affect cellular migration through their interactions with a variety of proteins, including kinases, integrins, actins, GTPase

activating proteins, and scaffolding proteins. As with adhesion signalling, the Src-FAK association is particularly important for migration; when FAK is activated following integrin stimulation it acts to recruit the SFKs to transient signalling complexes that induce migration through a number of signalling pathways that regulate the formation and disassembly of focal adhesions (Mitra and Schlaepfer, 2006). Indeed, the autophosphorylation of FAK on Tyr 397 and subsequent association with either Src or Fyn is required for cells that are overexpressing FAK to have increased migration on fibronectin (Cary *et al*., 1996). Interestingly, although the catalytic activity of Src has been shown to be necessary for migration in response to integrin signalling, the scaffolding functions of Src are not required (Cary *et al*., 2002). Indeed, the elevated expression and activity of Src and/or FAK in cancer cells likely leads to their increased migration by activating pathways that facilitate focal adhesion turnover.

Some of the Src-FAK substrates involved in migration are the scaffolding proteins $p130^{Cas}$ and paxillin, both of which are involved in cytoskeleton organization, adhesion, and cellular migration (Schaller, 2001; Huang *et al*., 2002), and cortactin, which is also involved in cytoskeleton organization (Figure 1.4) (Wu and Parsons, 1993; Huang *et al*., 1998; Huang *et al*., 2003). For example, Src-FAK activation has been found to result in the increased phosphorylation of FAK, paxillin, and $p130^{Cas}$ in response to vascular endothelial growth factor receptor (VEGFR) stimulation (Lesslie *et al*., 2006), while the expression of a kinase inactive Src results in decreased phosphorylation of paxillin and cortactin (Kilarski *et al*., 2003). As these adaptor proteins are involved in recruiting further downstream effector molecules that lead to focal adhesion turnover, the SFKs have an important role in the increased migratory capacity of cancer cells.

1.4.3.5. Src family kinase involvement in angiogenesis

The SFKs are also involved in angiogenesis, or the formation of new blood vessels from the existing vasculature. This is a multistep process that requires the proliferation, migration, differentiation, and invasion of endothelial cells, all of which are regulated, at least in part, by the SFKs (reviewed in Ferrara and Kerbel, 2005). The SFKs are involved in angiogenesis through two separate mechanisms (Figure 1.5). Firstly, they are involved in regulating the expression of angiogenic growth factors and cytokines such as VEGF (Summy *et al*., 2005) and interleukin (IL)-8 (Trevino *et al*., 2005) in tumour cells. The release of these pro-angiogenic

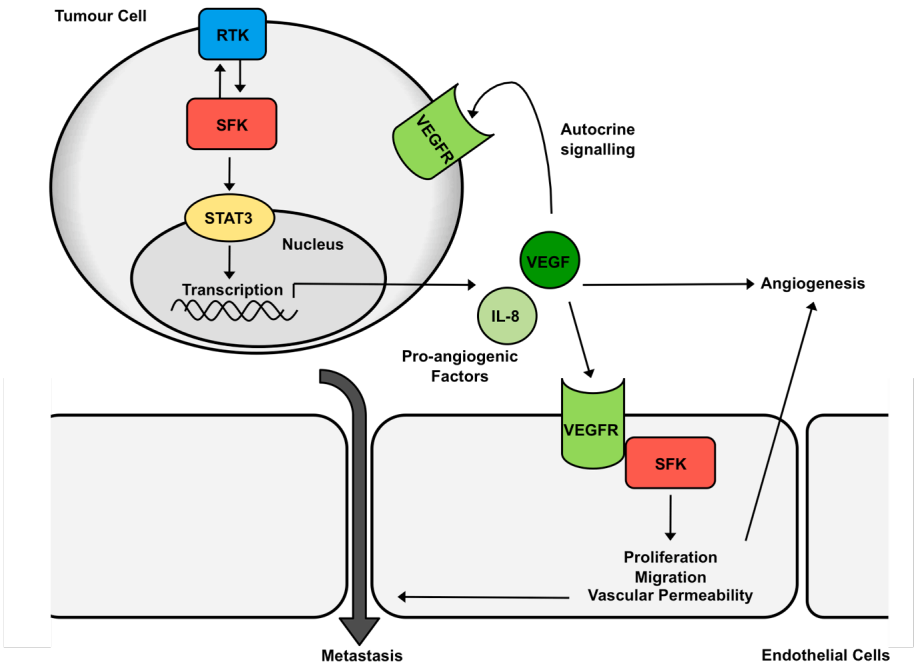


Figure 1.5. Src family kinase involvement in angiogenesis and metastasis. The SFKs are involved in regulating angiogenesis and metastasis through their effects on both tumour cells and endothelial cells. By activating transcription factors such as STAT3, activated SFKs promote the expression of pro-angiogenic factors such as VEGF and IL-8 in tumour cells. These factors are secreted by the tumour cells and are able to interact with receptors on the surrounding endothelial cells, as well as on the tumour cells themselves. This results in SFK activation, and the subsequent increased proliferation and migration of the endothelial cells results in the growth of blood vessels towards the tumour. In addition, the vascular permeability of the endothelial cell layer is increased as a result of this signalling, thereby facilitating metastasis. (Modified extensively from Kim *et al*., 2009b).

factors into the area surrounding the tumour subsequently results in the stimulation of the surrounding endothelial cells, which leads to the induction of capillary growth towards the tumour. Secondly, as the SFKs have a role in the signalling pathways that are initiated in response to these pro-angiogenic factors in both endothelial cells and the tumour cells themselves, they have a further role in this process. Therefore, the SFKs are involved not only in the production of pro-angiogenic factors but also in the cellular responses to these factors and, as such, play an important role in angiogenesis.

One of the best-characterized ways in which the SFKs are involved in angiogenesis is through the regulation of VEGF expression, particularly in response to hypoxia, or a lack of

oxygen (reviewed in Lesslie and Gallick, 2005). The SFKs regulate VEGF expression by activating the STAT transcription factors in response to RTK stimulation (Silva, 2004); STAT3 is a direct transcriptional activator of VEGF expression (Figure 1.5) (Niu *et al*., 2002; Wei *et al*., 2003). The expression of VEGF varies directly with the level of Src activity, whereby increased Src activity induces the upregulation of VEGF expression, while the repression of Src results in the downregulation of VEGF expression (Fleming *et al*., 1997). Therefore, as there is an increase in Src activity in response to hypoxia, there is a corresponding increase in VEGF expression that leads to angiogenesis (Mukhopadhyay *et al*., 1995b). Similarly, the expression of v-Src has been found to result in increased levels of VEGF being expressed in response to hypoxia (Mukhopadhyay *et al*., 1995a). Src has also been shown to be required for the induction of VEGF expression in response to hypoxic conditions in prostate and pancreatic cell lines (Gray *et al*., 2005), and in Müller cells (Werdich and Penn, 2006). Interestingly, there is no increase in Fyn or Yes activity in response to hypoxia, which suggests that Src may be the primary SFK involved in the regulation of VEGF expression (Mukhopadhyay *et al*., 1995b).

Several studies have also shown that decreased Src activity results in decreased VEGF expression within a cell. For instance, an antisense strategy targeting Src, but not Yes, has been found to result in decreases in both constitutive and hypoxia-induced VEGF expression in colon cancer cells (Ellis *et al*., 1998). VEGF expression was likewise inhibited in ovarian tumour cells expressing a Src antisense construct (Wiener *et al*., 1999). Similarly, the expression of either a dominant-negative Src or treatment with a tyrosine kinase inhibitor have been shown to result in decreased VEGF expression in response to hypoxia in U87 glioma cells and 293 kidney cells (Mukhopadhyay *et al*., 1995b). Finally, a subcutaneous mouse model of colon cancer formed using Src antisense clones has also been found to exhibit decreased vascularity when compared to that observed in control mice injected with parental cells, suggesting that Src is involved in regulating angiogenesis *in vivo* as well (Ellis *et al*., 1998). Cumulatively, these studies strongly support a role for Src in the expression of VEGF, and, as VEGF is secreted by many solid tumours and is important in mediating tumour angiogenesis, as well as the proliferation, migration, differentiation and vascular permeability of endothelial cells (Leung *et al*., 1989; Neufeld *et al*., 1999; Lesslie *et al*., 2006), the involvement of the SFKs in regulating its expression is a significant way in which they contribute to angiogenesis.

The second way in which the SFKs are involved in angiogenesis is through their role in signalling pathways initiated by the stimulation of angiogenic growth factor receptors, such as VEGFR, in both endothelial cells and tumour cells (Lesslie and Gallick, 2005; Kim *et al*., 2009b). When these receptors are activated on endothelial cells by growth factor or cytokine binding, the SFKs become activated, which leads to the initiation of signalling cascades that result in cellular proliferation and migration, as well as the formation of new blood vessels (Kim *et al*., 2009b). As VEGFRs are also expressed on the surface of tumour cells, it is likely that autocrine signalling through this receptor also plays a role in cancer progression, whereby the biological effects of Src activity are amplified in the tumour cell in response to VEGF stimulation (Figure 1.5) (Park *et al*., 2007). The SFKs are therefore important components of signalling pathways that can lead to angiogenesis in both tumour cells and the surrounding endothelial cells. Consequently, not only would SFK inhibition decrease the progression of primary tumours, but it would also impede signalling between the tumour cells and the surrounding cells by preventing the expression of pro-angiogenic factors by the tumours. As such, SFK inhibitors could be a valuable therapy for inhibiting tumour growth.

1.4.3.6. Src family kinase involvement in invasion and metastasis

Metastasis is the process whereby cancer cells detach from a primary tumour and move through the bloodstream, the lymphatic system, or the body cavity to a distant site within the body, in which they establish a secondary tumour. Since cancer cells must develop the ability to migrate and spread before they are able to metastasize, and the SFKs are integral to both the loss of stable cell-cell contacts and cell-ECM adhesion, as well as to migration, it is not surprising that the SFKs are involved in metastasis (Figure 1.4) (reviewed in Shah and Gallick, 2007). Indeed, the level of Src activity has been shown to correlate to the ability of tumour cells to metastasize. For instance, mice injected with breast cancer cells overexpressing a constitutively active Src developed significantly increased bone lesions, in both size and number of osteoclasts, while those overexpressing a dominant-negative mutant had reduced bone metastasis (Myoui *et al*., 2003). SFK activity has also been found to correlate with the metastatic potential of HuO9 osteosarcoma cells; Fyn in particular was found to have increased autophosphorylation in highly metastatic sublines of this cell line (Azuma *et al*., 2005). Therefore, the level of SFK activity within cancer cells impacts their metastatic potential.

SFK signalling in the endothelial cells surrounding a tumour has also been shown to be essential for metastasis. For instance, *src*$^{-/-}$ and *yes*$^{-/-}$ mice in which VEGF-expressing tumour cells have been injected intravenously have significantly fewer lung and liver metastases when compared with control mice (Weis *et al*., 2004). Similarly, *src*$^{-/-}$ mice have also been shown to have less tumour-induced vascular permeability and significantly fewer lung metastases following both subcutaneous and intravenous injection with lung tumour cells (Criscuoli *et al*., 2005). Interestingly, the subcutaneous growth of tumour cells was unaffected in the knockout mice, suggesting that although Src activity was not required for tumour cell growth, its activity in endothelial cells is necessary for metastasis (Criscuoli *et al*., 2005). Taken together, these studies indicate that the SFKs need to be active in the endothelial cells surrounding a tumour for metastasis to occur. As there is increased space between endothelial cells following SFK activation, this is likely due to their involvement in inducing the increased vascular permeability of the endothelial cell layer, which allows tumour cells to intravasate more easily into the vascular space (Figure 1.5) (Kim *et al*., 2009a). Following the dissemination of the tumour cells through the vascular system, the extravasation from the vessels at a secondary site where they are able to establish metastases is also facilitated by increased vascular permeability (Kim *et al*., 2009a).

Further to decreasing adhesion and increasing the migratory capacity of cancer cells, the SFKs also contribute to metastasis through upregulating the expression of proteinases. For instance, FAK-Src signalling contributes to an invasive phenotype by activating p130^{Cas}, which results in the induction and activation of the matrix metalloproteinases MMP-2 and MMP-9 (Van Slambrouck *et al*., 2007). The upregulation of these proteinases facilitates invasion by degrading the basement membrane. Therefore, the SFKs are involved in promoting invasion and metastasis in multiple ways, further supporting their importance in later stages of tumour progression.

1.5. SRC FAMILY KINASE INHIBITORS

As the SFKs are involved in a number of different pathways important in tumour progression, they are an attractive target for the treatment of cancer. In particular, cancers that have very poor prognoses and high mortality, such as pancreatic cancer, could benefit from SFK inhibition. Efforts have therefore been made in order to develop clinically relevant

chemical inhibitors capable of inhibiting the SFKs. Although the first SFK inhibitors, such as herbimycin A and genistein, were non-specific, a great deal of progress has been made and several SFK inhibitors are currently in clinical trials (Wheeler *et al.*, 2009; Lieu and Kopetz, 2010; Saad and Lipton, 2010).

One major hurdle to the use of SFK inhibitors as a cancer therapy is the specificity, or lack thereof, of these drugs. As all protein tyrosine kinases share similar SH1, SH2, and SH3 domain structures, inhibitors developed targeting these regions often have significant off-target effects on other tyrosine kinases. For instance, the clinically relevant SFK inhibitors dasatinib and 4-Anilino-3-quinolinecarbonitrile (SKI-606) inhibit Abl kinase in addition to the SFKs, while dasatinib also inhibits EGFR (Cao *et al*., 2008). Another significant problem with cancer treatment is the development of resistance to anti-cancer drugs. The SFKs have themselves been implicated in this phenomenon; therefore, the use of SFK inhibitors in combination with other chemotherapeutic agents has promise in cancer therapy, as this would prevent the activation of the SFKs in response to other chemotherapy drugs. SFK inhibition may therefore prove to be a particularly valuable cancer therapy.

1.5.1. Structures, targets and clinical trials

The most effective SFK inhibitors are ATP mimics that are competitive for the ATP binding sites of the SFKs. Compounds with several different types of scaffolding structures have been found to inhibit the SFKs in this fashion, with differing levels of specificity; pyrazolopyrimidines (Hanke *et al*., 1996), pyrrolopyrimidines, pyrido-pyrimidinones, indolinones, naphthyridinones, aminopyridopyrimidinyl urea, quinazolines and quinolines are all capable of inhibiting the SFKs (reviewed in Tsygankov and Shore, 2004). All of the ATP-binding site inhibitors have a heteroaromatic ring system that is capable of hydrophobic interactions with the kinase domain (Cao *et al*., 2008). Although the kinase domains of different proteins are somewhat conserved, differences in these domains allow chemical inhibitors to inhibit particular tyrosine kinases more efficiently than others. For instance, adjacent to the ATP binding site in the kinase domain is a hydrophobic pocket. As the structure of the hydrophobic pocket varies between kinases, and some small molecules, including SFK inhibitors, are able to bind both the hydrophobic pocket and the ATP binding site, this provides one way in which these inhibitors are selective (Chong *et al*., 2005). Even so, chemical

inhibitors are not completely specific due to similarities in the ATP-binding site of different kinases, and often have off-target effects. For instance, some SFK inhibitors are unable to specifically inhibit a given SFK; rather, they can inhibit the kinase activity of multiple family members (Hanke *et al*., 1996; Liu *et al*., 1999; Blake *et al*., 2000).

One of the most widely used SFK inhibitors in preclinical research is 4-amino-5-(4-chorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) (Figure 1.6). This compound, along with 4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1), was originally found to be an ATP-competitive inhibitor selective for the SFKs (Hanke *et al*., 1996). However, these compounds have also been found to inhibit other tyrosine kinases including the EGF receptor and Janus Kinase 2 (Jak2) (Chen *et al*., 2006). The other SFK inhibitor most commonly used in preclinical studies is 2-Oxo-3(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide (SU6656) (Figure 1.6). This inhibitor is another ATP-competitive small molecule inhibitor that inhibits Src, Fyn, Yes, and Lyn, although it is a poor inhibitor of Lck. SU6656 does not, however, significantly inhibit PDGFR, which allows it to be used to investigate SFK involvement in the pathways induced in response to the stimulation of this receptor (Blake *et al*., 2000). In addition to these two SFK inhibitors, two other commercially available SFK inhibitors have been used in the studies discussed in this thesis; namely, 4-(4'-phenoxyanilino)-6.7-dimethoxyquniazoline, or Src Kinase Inhibitor I (SKI I) and the substituted triazoloquinoline-1-thione compound Src Kinase Inhibitor II (SKI II), which are available from Merck (Figure 1.6). SKI I interacts with both the peptide binding site and the ATP binding site of multiple SFKs, and also inhibits VEGFR and c-fms at higher concentrations (Tian *et al*., 2001), while SKI II inhibits both the SFKs and Csk (Kilimnik *et al*., 2003). Therefore, although SFK inhibitors also inhibit proteins other than the SFKs, their non-specific effects are dependent upon the particular SFK inhibitor being used.

There are several SFK inhibitors currently in clinical trials or in use as cancer therapies. For example, the SFK inhibitor dasatinib, from Bristol-Myers Squibb, is an orally available chemical inhibitor of several SFKs, as well as Bcr-Abl, and is currently the only SFK inhibitor approved for the treatment of chronic myelogenous leukemia (CML) and Philadelphia positive acute lymphoblastic leukemia (Wheeler *et al*., 2009). In addition, there are also phase I and II clinical trials currently underway examining the use of this drug in non-Hodgkin's lymphoma, and refractory leukemia in adolescents, as well as in metastatic solid cancers including breast

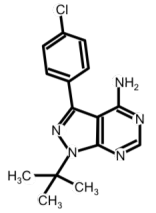
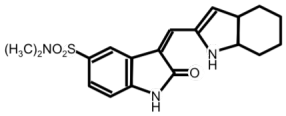
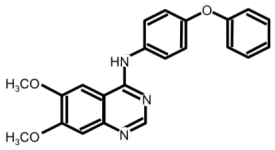
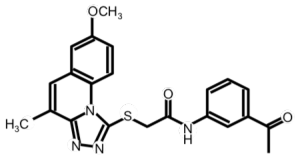
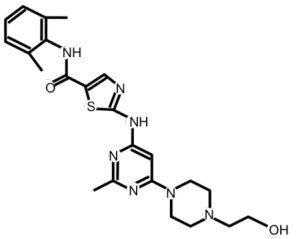
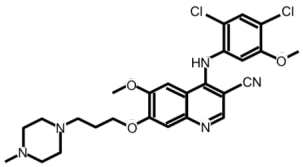
Name	Structure	Targets	Current Clinical Trials
PP2		SFKs, EGFR, Jak2	——
SU6656		SFKs (poor Lck inhibitor)	——
SKI I		SFKs, VEGFR, c-fms	——
SKI II		SFKs, Csk	——
Dasatanib		SFKs, Bcr-Abl, Kit, Eph, receptors, PDGFR	Use in CML, Phase I and II trials in solid cancers
SKI-606		SFKs, Bcr-Abl	Phase I, II, and III clinical trials in solid cancers

Figure 1.6. The structures of selected Src family kinase inhibitors. PP2, SU6656, SKI I, and SKI II are available commercially, while Dasatinib and SKI-606 are clinically relevant Src kinase inhibitors currently undergoing clinical trials.

and prostate cancers (Wheeler *et al*., 2009). Dasatinib is of particular interest as it has been found to have minimal effects on normal hematopoietic cells (Alvarez *et al*., 2006). SKI-606 (Figure 1.6), also known as bosutinib, is also an orally available, dual Src-Abl kinase inhibitor, produced by Wyeth Pharmaceuticals, Inc., that is capable of inhibiting multiple SFKs (Alvarez *et al*., 2006). Like dasatinib, there are currently several clinical trials underway in order to investigate the use of SKI-606 in the treatment of a variety of cancers, including those that are unresponsive to standard treatments, as well as metastatic cancers (Wheeler *et al*., 2009). As some of the cancers being investigated are difficult to treat using current chemotherapeutic drugs, the development of effective SFK inhibitors would be of great use.

1.5.2. Preclinical studies of clinically relevant Src family kinase inhibitors

A variety of SFK inhibitors have been used to examine the effect of SFK inhibition on various cancer cell phenotypes in preclinical studies. Similar to what has been observed in other investigations into the involvement of the SFKs in cellular processes in cancer cell lines, studies using SFK inhibitors have found the SFKs to be primarily involved in migration and invasion. For instance, the SFK inhibitor PP2, which has been used extensively in preclinical investigations of the SFKs, has been shown to decrease the ability of breast cancer cells to form colonies in soft agar, as well as tumours in nude mice (Ishizawar *et al*., 2004). This was supported by similar findings when cells overexpressing a catalytically inactive Src were used (Ishizawar *et al*., 2004). Clinically relevant SFK inhibitors have also shown effects when used in cell culture. For instance, the treatment of melanoma cells (Buettner *et al*., 2008), bone and soft tissue sarcoma cell lines (Shor *et al*., 2007), malignant pleural mesothelioma cell lines (Tsao *et al*., 2007), and prostate cancer cell lines (Nam *et al*., 2005; Park *et al*., 2008) with dasatinib results in an inhibition of adhesion, migration, and invasion. Dasatinib treatment has also been shown to result in the increased apoptosis and decreased invasion of glioblastoma cells, which was confirmed by silencing the expression of either Src or Fyn (Lu *et al*., 2009). Similarly, the treatment of breast cancer cell lines with SKI-606 also results in the decreased motility and invasive potential of these cells (Vultur *et al*., 2008). Therefore, clinically relevant SFK inhibitors have significant effects on the adhesion, migration, and colony forming ability of various cell lines *in vitro*.

In contrast to the effects of various SFK inhibitors on migration and invasion, the effects of SFK inhibitor treatment on cellular proliferation are varied, and SFK inhibition does not impact the cellular proliferation of certain cell lines. For instance, the proliferation and survival of melanoma cells was found to be unaffected by treatment with dasatinib (Buettner *et al*., 2008). Dasatinib treatment also did not affect the proliferation of ten of twelve colon cancer cell lines tested in another study (Serrels *et al*., 2006). Similarly, despite its ability to inhibit migration and invasion, SKI-606 did not affect either the proliferation or survival of selected breast cancer cell lines (Vultur *et al*., 2008), nor did it have an antiproliferative effect on HT29 or Colo 205 colon cancer cell lines (Golas *et al*., 2005). However, the proliferation of some cell lines has been reported to be reduced following SFK inhibitor treatment. For instance, the SFK inhibitor PD173955 causes an arrest in the mitotic progression of a variety of cancer cell lines (Moasser *et al*., 1999), while SKI-606 has been found to inhibit the proliferation of CML cells in culture (Golas *et al*., 2003). Finally, the inhibition of Lyn with a sequence based peptide inhibitor in hormone-refractory prostate cancer cell lines has also been shown to decrease the proliferation of these cells (Goldenberg-Furmanov *et al*., 2004). Therefore, the involvement of the SFKs in cellular proliferation may be somewhat cell specific.

SFK inhibitor treatment has also been found to impact tumour growth *in vivo*. In a study investigating the effects of dasatinib on prostate cancer growth, both tumour growth and the development of lymph node metastasis were inhibited in an orthotopic nude mouse model (Park *et al*., 2008). SKI-606 has also been shown to be effective in decreasing tumour growth (Golas *et al*., 2005); daily oral administration of this drug to mice bearing xenografts of the CML cell line K562 resulted in the complete regression of the xenografts (Golas *et al*., 2003). Similarly, the inhibition of Lyn kinase using a peptide inhibitor has also been found to result in both apoptosis and tumour regression in nude mice bearing tumours formed from prostate cancer cell lines (Goldenberg-Furmanov *et al*., 2004). Together, these preclinical studies demonstrate that SFK inhibition has significant effects on multiple cancer cell phenotypes, further supporting a potential clinical benefit of using SFK inhibitors to treat cancer.

1.5.3. Drug resistance

A portion of a cancer cell population may develop resistance to chemotherapeutic agents following their extended use. This is a serious problem, as it results in the expansion of a

population of cells that are able to evade the toxic effects of these previously effective drugs. The SFKs have been implicated in the development of chemoresistance of both solid cancers and CML (Shah and Gallick, 2007). For instance, resistance to various anticancer agents such as gleevec, gemcitabine, and taxol has been found to correlate with increased Src activation, suggesting that SFK inhibitors may be particularly useful in cancer therapy (Summy and Gallick, 2006). Src activity also correlates directly with pancreatic cancer cell chemoresistance to gemcitabine; inhibiting Src in these cells results in an increase in gemcitabine-induced apoptosis (Duxbury *et al*., 2004a). This was further supported by the finding that treatment of these pancreatic cancer cells with siRNA directed against Src likewise resulted in increased gemcitabine sensitivity (Duxbury *et al*., 2004b). Fyn has also been found to have a role in imatinib resistance, as both PP2 treatment and siRNA directed against Fyn result in a re-sensitization of CML cells to imatinib (Grosso *et al*., 2009). As the use of SFK inhibitors in conjunction with other cancer drugs sensitizes the target cells to these agents, combined therapies using SFK inhibitors may be more effective than current treatments as they could result in greater initial cell death, and thereby prevent, or at least postpone, the development of a resistant phenotype.

The usefulness of SFK inhibitors in combined therapies has been demonstrated both *in vitro* and *in vivo*. The drug 5-fluorouracil (5-FU) is commonly used to treat different types of cancer such as pancreatic and colon cancer; however, as with other cancer drugs, the development of resistance to 5-FU is a problem. As the treatment of 5-FU resistant pancreatic cancer cells with a combination of 5-FU and PP2 has a synergistic effect on the ability of 5-FU to induce apoptosis in these cells, it suggests that SFK inhibitors could be useful for combating chemoresistance (Ischenko *et al*., 2007). *In vivo* tumour growth and metastasis were also decreased in mice treated with a combination of PP2 and 5-FU (Ischenko *et al*., 2007). Similarly, treatment of a mouse model of colorectal liver metastasis with dasatinib combined with the cytotoxic drug oxaliplatin has also been found to result in synergistic effects leading to decreased tumour growth (Kopetz *et al*., 2009). Indeed, inhibiting the SFKs in conjunction with other targets appears to be able to sensitize cancer cells to these drugs, leading to cell death. Therefore, the use of SFK inhibitors in conjunction with other therapies, such as other chemotherapeutic drugs, radiation, or surgery, could lead to more successful cancer treatments.

2. SPECIFIC AIMS AND HYPOTHESIS

Given that a number of the SFKs are overexpressed and activated in a variety of different cancers and cancer cell lines, it is likely that these proteins have redundant or complementary functions in regards to cancer cell phenotypes. In particular, the ubiquitously expressed SFKs, Src, Fyn, and Yes, are often co-expressed. However, studies often only investigate the involvement of Src in cancer cell phenotypes by either specifically increasing or decreasing the level of Src expression, and do not examine the involvement of the other SFKs. Conversely, while a large number of studies have used SFK inhibitors to examine the effects of SFK inhibition on a variety of cancer cell phenotypes, this approach results in the inhibition of multiple SFKs. This prevents the involvement of the individual SFKs in cellular processes from being elucidated. Therefore, although many of the SFKs have been implicated in various cancers, the involvement of the individual SFKs in given cancer cell phenotypes is not clear. It is of interest to identify which SFKs in particular are involved in these processes, as this could provide valuable insight into the suitability of these proteins for more targeted cancer therapies.

HYPOTHESIS: Multiple Src family members contribute to cancer cell phenotypes such as cellular proliferation, adhesion, migration, and tumour formation, within a given cell line.

SPECIFIC AIMS:

1. To examine the expression and activity of the individual SFKs in a wide panel of human cancer cell lines.

2. To use a systematic approach to investigate the role of the SFKs in the cellular proliferation, adhesion, migration, and colony forming ability of four selected cancer cell lines.

3. To compare the changes in proliferation, adhesion, migration, and colony forming ability that result from inhibiting multiple SFKs using SFK inhibitors to those resulting from RNAi-mediated decreases in the expression of Src, Fyn, and Yes.

3. MATERIALS AND METHODS

3.1. Reagents, Kits, Equipment and Distributors

Commercially available reagents and kits were used in the studies described in this thesis. These products and their respective distributors are outlined in the following tables: Table 3.1. Reagents, Table 3.2. Kits, Table 3.3. Equipment and Software, Table 3.4. Antibodies. The addresses of the distributors used may be found in Table 3.5.

Table 3.1. Reagents and Distributors

Reagent	Distributor Name
[α-32P] dCTP	PerkinElmer Life Sciences Inc.
Acetic acid (glacial)	BDH
2-Butanol (water saturated)	Sigma-Aldrich Co.
Acetic acid (glacial)	BDH
Acrylamide	EM Sciences
Actinomycin D	Sigma-Aldrich Co.
Agarose	EMD Chemicals Inc.
Ammonium persulfate	EM Sciences
Ampicillin	EMD Chemicals Inc.
ATP	Sigma-Aldrich Co.
β-glycerophosphate	Sigma-Aldrich Co.
β-mercaptoethanol	EMD Chemicals Inc.
Bacto agar	BD Biosciences
BioMax XAR film	Kodak
Biotrace NT membrane	Pall
Bis-acrylamide	EM Sciences
Boric acid	Sigma-Aldrich Co.
Bovine serum albumen	EMD Chemicals Inc.
Bromophenol blue	BDH
Calcium chloride dihydrate	BDH
Calf Intestinal Alkaline Phosphatase	New England Biolabs Ltd.
Carbenicillin	EMD Chemicals Inc.
CellTitre 96 Aqueous One Solution	Promega
Chloroform	EMD Chemicals Inc.
Collagen	Invitrogen

Crystal violet	Sigma-Aldrich Co.
Diethyl pyrocarbonate	Sigma-Aldrich Co.
Disodium hydrogen phosphate	EMD Chemicals Inc.
DL-Dithiothreitol	Sigma-Aldrich Co.
DMEM	Invitrogen – Gibco Cell Culture Systems
DMEM-F12	Invitrogen – Gibco Cell Culture Systems
DMSO	Sigma-Aldrich Co.
dNTPs	New England Biolabs Ltd.
EDTA	EMD Chemicals Inc.
Ethanol	EMD Chemicals Inc.
Ethidium bromide	BDH
Express Hyb	BD Biosciences
Fat-free skim milk powder	Carnation
Fetal Bovine Serum	HyClone
Fibronectin	Sigma-Aldrich Co.
Formaldehyde	EMD Chemicals Inc.
Formamide	BDH
Fyn EST (7997662)	ATCC
GeneScreen Plus Hybridization Transfer Membrane	PerkinElmer Life Sciences, Inc.
Glycerol	EMD Chemicals Inc.
Glycine	EMD Chemicals Inc.
Guanidinium thiocyanate	BDH
Hexadimethrine bromide	Sigma-Aldrich Co.
Humulin	Eli Lilly Canada Inc.
Hydrochloric acid	EMD Chemicals Inc.
Insulin-Transferrin -Selenium-G Supplement	Invitrogen--Gibco Cell Culture Systems
Isoamyl alcohol	BDH
Isopropanol	EMD Chemicals Inc.
Lck EST (6883710)	ATCC
Lipofectamine	Invitrogen
Lipofectamine 2000	Invitrogen
Lipofectamine RNAiMAX	Invitrogen
Lyn EST (5509761)	ATCC
Magnesium chloride	BDH
Manganese chloride tetrahydrate	BDH
McCoy's 5A	Invitrogen – Gibco Cell Culture Systems
Methanol	EMD Chemicals Inc.
Molecular Imager FX Imaging Screen K	Bio-Rad Laboratories Ltd.
Morpholinopropanesulfonic acid	Sigma-Aldrich Co.
N-lauroyl sarcosine	Sigma-Aldrich Co.

N,N,N',N'-Tetramethylethylenediamine	EMD Chemicals Inc.
Odyssey Blocking Buffer	Li-Cor Biosciences
Opti-MEM I Reduced Serum Medium	Invitrogen--Gibco Cell Culture Systems
Paraformaldehyde	Sigma-Aldrich Co.
Penicillin-Streptomycin	Invitrogen – Gibco Cell Culture Systems
Phenol (water saturated)	EMD Chemicals Inc.
Potassium acetate	BDH
Potassium chloride	EMD Chemicals Inc.
Potassium dihydrogen phosphate	EMD Chemicals Inc.
PP2	EMD Chemicals Inc.
Prestained Protein Marker	Fermentas
Protease Inhibitor Cocktail	Sigma-Aldrich Co.
Protein G Agarose, Fast Flow	Millipore
Puromycin	Sigma-Aldrich Co.
Restriction endonucleases	New England Biolabs Ltd.
RPMI–1640	Invitrogen – Gibco Cell Culture Systems
Rubidium chloride	EMD Chemicals Inc.
Sodium acetate	BDH
Sodium bicarbonate	EMD Chemicals Inc.
Sodium carbonate	EMD Chemicals Inc.
Sodium chloride	EMD Chemicals Inc./Sigma
Sodium citrate	BDH
Sodium deoxycholate	Sigma-Aldrich Co.
Sodium dihydrogen phosphate	BDH
Sodium dodecyl sulfate	Sigma-Aldrich Co.
Sodium hydroxide	BDH
Sodium orthovanadate	Sigma-Aldrich Co.
Sodium phosphate (dibasic)	EMD Chemicals Inc.
Sodium phosphate (monobasic)	EMD Chemicals Inc.
Src Kinase Inhibitor I	EMD Chemicals Inc.
Src Kinase Inhibitor II	EMD Chemicals Inc.
SU 6656	EMD Chemicals Inc.
Tris	EMD Chemicals Inc.
Triton X-100	BDH
Trypsin-EDTA 1X	Invitrogen – Gibco Cell Culture Systems
Tween 20	Sigma-Aldrich Co.
UV Stratalinker 2400	Stratagene
Western Lightning Chemiluminescence Reagent Plus	PerkinElmer Life Sciences Inc.
X-OMAT Blue XB- film	Kodak
Yes EST A50(7497767)	ATCC

Table 3.2. Commercial Kits and Distributors

Commercial Kit	Distributor Name
EndoFree Plasmid Maxi Kit	Qiagen Inc.
QIAquick Gel Extraction Kit	Qiagen Inc.
QIAquick Nucleotide Removal Kit	Qiagen Inc.
Ready-To-Go DNA Labeling Beads (-dCTP)	GE Healthcare
Total Protein Kit, Micro Lowry	Sigma
Tyrosine Kinase Activity Assay	Millipore

Table 3.3. Equipment and Software and Distributors

Equipment and Software	Distributor Name
Biofuge 13 microcentrifuge	Thermo Electron Corporation - Heraeus
CO2 Incubator 3326	Forma Scientific, Inc.
Coolpix 990 Digital Camera	Nikon
Coulter Counter ZM	Coulter Electronics, Ltd.
Eclipse TE300	Nikon
Gel Doc 2000	Bio-Rad Laboratories Ltd.
Gene Amp PCR System 2700	Applied Biosystems Canada
Horizontal Gel Electrophoresis System	Owl Separation Systems
IEC Micromax RF	Fisher Scientific
ImageJ Software	National Institutes of Health
Isotemp Incubator 230D	Fisher Scientific Company
J2-MI highspeed centrifuge	Beckman Coulter Canada, Inc.
JA-10 rotor	Beckman Coulter Canada, Inc.
Micro Hybridization Incubator Model 2000	Robbins Scientific
Microplate Reader Model 3550	Bio-Rad Laboratories Ltd.
Mini-PROTEAN II Cell	BioRad Laboratories Ltd.
Molecular Imager FX	Bio-Rad Laboratories Ltd.
Odyssey Infrared Imaging System	Li-Cor Biosystems
Odyssey Software	Li-Cor Biosystems
ORBIT Incubator Shaker	Lab-Line
Quantity One Software, Version 4	Bio-Rad Laboratories Ltd.
Semi-dry Transfer Apparatus	Owl Separation Systems
SmartSpec 3000 Spectrophotometer	Bio-Rad Laboratories Ltd.
Sorvall H1000B	Du Pont Canada, Inc.
Sorvall RT6000D	Du Pont Canada, Inc.
SpectraMax 340 PC	Molecular Devices Corporation
UV Stratalinker 2400	Stratagene

Table 3.4. Antibodies and Distributors

Wn, Western Blot; IP, Immunoprecipitation; Wn 2°, Western Blot secondary antibody

Antibody	Type	Distributor	Catalogue Number	Use
v-Src (Ab-1)	Mouse monoclonal (327)	Calbiochem	OP07	Wn, IP
Yes	Rabbit polyclonal IgG	Upstate	06-514	Wn
c-Yes (C-4)	Rabbit polyclonal IgG	Santa Cruz	sc-14	IP
Fyn (15)	Mouse monoclonal IgG_1	Santa Cruz	sc-434	Wn
Fyn (FYN-01)	Mouse monoclonal IgG	Santa Cruz	sc-51598	Wn
Fyn (FYN3)	Rabbit polyclonal IgG	Santa Cruz	sc-16	IP
Lyn (H-6)	Mouse monoclonal IgG_{2a}	Santa Cruz	sc-7274	Wn, IP
Lck (3A5)	Mouse monoclonal IgG_{2b}	Santa Cruz	sc-433	Wn, IP
Actin (Ab-1)	Mouse monoclonal (JLA20)	Calbiochem	CP01	Wn
Goat anti Mouse	IgG HRP conjugated	Santa Cruz	sc-2005	Wn 2°
Goat anti Rabbit	IgG HRP conjugated	Santa Cruz	sc-2004	Wn 2°
Goat anti Mouse	IRDye 800 CW	Li-Cor Biosystems	926-32210	Wn 2°
Goat anti Rabbit	IRDye 800 CW	Li-Cor Biosystems	926-32211	Wn 2°
Goat anti Rabbit	IRDye 680	Li-Cor Biosystems	926-32221	Wn 2°

Table 3.5. siRNA and shRNA Sequences and Distributors

siRNA or shRNA	Sequence	Distributor	Catalogue Number
Silencer Negative Control siRNA #1	Proprietary	Applied Biosystems Canada	AM51331
siGENOME Control siRNA Non-Targeting siRNA #2	Proprietary	Dharmacon	D-001210-02-05
Src siGENOME Duplex	CGUCCAAGCCGC AGACUCAUU	Dharmacon	D-003175-06
Fyn siGENOME Duplex	GGAAUGGACUCA UAUGCAAUU	Dharmacon	D-003140-05
Yes Silencer Validated siRNA 690	Proprietary	Applied Biosystems Canada	AM51331
pGIPZ Lentiviral shRNAmir	None	Open Biosystems	RHS4430
Src shRNA Lentiviral Vector	CTCATCATAGCA ATAACAT	Open Biosystems	RHS4430-98524478
Fyn shRNA Lentiviral Vector	GAGACCATGTCA AACATTA	Open Biosystems	RHS4430-99365271
Yes shRNA Lentiviral Vector	GTGACAATGTGA AACACTA	Open Biosystems	RHS4430-98820654

Table 3.6. Names and Addresses of Distributors

Distributor Name	Address
American Type Culture Collection (ATCC)	ATCC, Manassas, VA, USA
Applied Biosystems Canada	Applied Biosystems Canada, Streetsville, ON, Canada
BD Biosciences	BD Biosciences, Mississauga, ON, Canada
BDH Inc.	BDH Inc., Toronto, ON, Canada
Beckman Coulter Canada, Inc.	Beckman Coulter Canada, Inc., Mississauga, ON, Canada
Bellco Glass	Bellco Glass, Vineland, NJ, USA
Bio-Rad Laboratories Ltd.	Bio-Rad Laboratories Ltd., Mississauga, ON, Canada
Calbiochem	EMD Biosciences Inc., La Jolla, CA, USA
Coulter Electronics Ltd.	Coulter Electronics Ltd. Luton, Beds., England
Dharmacon	Thermo Scientific, Dharmacon, Inc., Lafayette, CO, USA
Du Pont Canada, Inc.	Du Pont Canada, Inc., Mississauga, ON, Canada
Eli Lilly Canada Inc.	Eli Lilly Canada Inc., Scarborough, ON, Canada
EMD Chemicals Inc.	EMD Chemicals Inc., Gibbstown, NJ, USA
Eppendorf AG	Eppendorf AG, Hamberg, Germany
Fermentas Canada Inc.	Fermentas Canada Inc., Burlington, ON, Canada
Fisher Scientific Company	Fisher Scientific Company, Nepean, ON, Canada
Forma Scientific, Inc.	Forma Scientific, Inc., Marietta, OH, USA
GE Healthcare Bio-Sciences Inc.	GE Healthcare Bio-Sciences Inc., Baie d'Urfe, QC, Canada
HyClone	HyClone, Logan, UT, USA
Invitrogen Canada Inc.	Invitrogen Canada Inc., Burlington, ON, Canada
Kodak	Kodak Eastman Company, Rochester, NY, USA
Millipore	Millipore, Billerica, MA, USA
New England Biolabs, Ltd.	New England Biolabs, Ltd., Pickering, ON, Canada
Open Biosystems	Thermo Scientific Open Biosystems, Huntsville, AL, USA
Owl Separation Systems	Owl Separation Systems, Portsmouth, NH, USA
Pall Canada, Ltd.	Pall Canada Ltd., Ville St. Laurent, QC, Canada
PerkinElmer Life Sciences, Inc.	PerkinElmer Life Sciences, Inc., Boston, MA, USA
Promega	Promega, Madison, WI, USA

Qiagen Inc.	Qiagen Inc., Mississauga, ON, Canada
Roche Diagnostics	Roche Diagnostics, Laval, QC, Canada
Santa Cruz	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Sigma-Aldrich Co.	Sigma-Aldrich Co., Oakville, ON, Canada
Stratagene	Stratagene, La Jolla, CA, USA
Thermo Electron Corporation - Heraeus	Thermo Electron Corporation, Waltham, MA, USA
US Biomax, Inc.	US Biomax, Inc., Rockville, MD, USA

3.2. Tissue Culture and Cell Lines

3.2.1. Cell lines and standard tissue culture conditions

All cell lines used throughout these studies were obtained from the American Type Culture Collection (ATCC) and grown at 37° in a Forma Scientific CO_2 incubator, model 3326, with 5% CO_2. The four cell lines used extensively in this work are all adherent epithelial cancer cell lines: HT29 colorectal adenocarcinoma cells, HCT116 colorectal carcinoma cells, SW480 Dukes' type B colorectal adenocarcinoma cells, and HepG2 hepatocellular carcinoma cells. The colon cancer cell lines Colo 201, Colo 205, Colo 320, DLD-1, and HCT-15 were grown in RPMI-1640 media containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), while HCT116 cells were grown in McCoy's 5A medium with 10% FBS and 1% P/S. HT29, KM12C, LS174 T, LS180, SW480, SW620 and WiDr colon cancer cell lines were all grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and 1% P/S. LoVo colon cancer cells were grown in DMEM F12 media with 10% FBS and 1% P/S. The breast cancer cell line Hs578T was grown in DMEM, while MDA-MB 231 and MDA-MB 435 cells were grown in RPMI-1640 and T47D cells were grown in RPMI-1640 medium supplemented with 0.2 units/mL humulin. All media for the breast cancer cell lines contained 10% FBS and 1% P/S. The HepG2 cell line was grown in DMEM or DMEM-F12, while the myeloid cell line U937 was grown in RPMI-1640, all containing 10% FBS and 1% P/S. The normal lung cell lines CCD-16Lu and CCD-19Lu were both maintained in α-MEM supplemented with 2 mM L-glutamine and Earle's balanced salt solution, 10% FBS and 1% P/S. The lung cancer cell lines were maintained as follows: HCC827, NCI-H1395, NCI-H727 and NCI-H23 in RPMI-1640 containing 4.5 g/L glucose and NCI-H1993 in RPMI-1640

with no additional supplements. NCI-H2009 cells were grown in DMEM-F12 supplemented with 5 μg/mL insulin, 0.01 mg/mL transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β-estradiol, 5% FBS and 1% P/S. All other lung cell lines were maintained in media containing 10% FBS and 1 % P/S. The HEK 293T cells used for viral packaging were maintained in DMEM containing 10% FBS. In order to expand or seed adherent cells, they were first washed with phosphate-buffered saline (PBS) (8 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), 1.44 g disodium hydrogen phosphate (Na_2HPO_4), 0.24 g potassium dihydrogen phosphate (KH_2PO_4) in 1 L water), detached from their culture dishes using Trypsin-ethylenediamine tetracetic acid (EDTA) and split at a ratio consistent with that recommended by ATCC. Cells used in proliferation, migration, adhesion, and colony forming assays were grown to at least 75 % confluency prior to seeding, in order to maintain consistency.

3.2.2. Src family kinase inhibitor treatments

Cells were trypsinized and seeded into plates a minimum of 24 hours prior to treatment with SFK inhibitors or the control DMSO. The SFK inhibitors were mixed into culture media at the indicated concentrations prior to treating the cells. To treat, cells were washed once with sterile PBS, following which the culture media containing the SFK inhibitors was added to the cells.

3.3. siRNA Transfection of Cultured Cells

3.3.1. Standard forward transfection conditions

Briefly, 24 hours before transfection HepG2 cells were trypsinized and counted using a Coulter Counter ZM and 5.0 x 10^5 cells seeded per well of a 24 well plate in media containing FBS but without P/S. Transfection mixes were made by first diluting the indicated concentration of siRNA in 50 μL OPTI-MEM serum free media for transfection in 24 well plates. Five μL of Lipofectamine 2000 was added to equivalent volumes of OPTI-MEM in separate sterile tubes and allowed to incubate at room temperature for 15 minutes before the addition of the siRNA dilutions. The mixtures were gently mixed by pipetting up and down and allowed to incubate for a further 15 minutes. The media on the cultured cells was changed to fresh media containing FBS prior to the transfection mixes being added dropwise to the wells.

To transfect cells in different sizes of well volumes were adjusted accordingly. The siRNAs commonly used in these studies may be found in Table 3.5.

3.3.2. Standard reverse transfection conditions

Transfection mixes were made up directly in the tissue culture plates being used. In general, 50 nM siRNA was mixed into 500 μL OPTI-MEM media in a 35 mm well, followed by 5 μL Lipofectamine RNAiMAX. The transfection mix was allowed to incubate for 15 to 20 minutes prior to the addition of cells. Cells were trypsinized and counted using a Coulter Counter ZM and 6.0 x 10^5 HepG2 cells seeded directly into the wells containing transfection mix so that they would be approximately 50% confluent the following day. Untreated control wells without siRNA or transfection reagent, as well as mock transfected wells (containing no siRNA), were also seeded at the same time.

3.4. Creation of shRNA Knockout Cell Lines

3.4.1. Preparation of competent Top10 cells

Top10 *Escherichia coli* cells were grown in 5 mL Luria Broth (LB) overnight at 37°C with shaking. This whole culture was used to inoculate 100 mL of LB and the culture was grown until an optical density (OD) 550 nm of approximately 0.4 was reached before being centrifuged at 1300 x g for 15 minutes at 4°C. The pellet was subsequently resuspended in 30 mL of RF1 Buffer, pH 5.8 (100 mM rubidium chloride (RbCl), 50 mM manganese chloride tetrahydrate ($MnCl_2$-$4H_20$), 30 mM potassium acetate (KOAc), 10 mM $CaCl_2$ dihydrate ($CaCl_2$-$2H_2O$), 15% (w/v) glycerol). Following a 15 minute incubation on ice the cells were recentrifuged and resuspended in 7.2 mL RF2 Buffer, pH 6.8 (10 mM N-morpholinopropanesulfonic acid (MOPS), 10 mM RbCl, 75 mM $CaCl_2$-$2H_20$, 75 mM glycerol). Cells were again incubated on ice for 15 minutes, aliquoted and flash frozen. Cells were stored at -80°C until use.

3.4.2. Transformation of competent Top10 cells

Top10 supercompetent cells were transformed with the lentiviral packaging vectors psPAX2 and pMD2.G or the control short hairpin RNA (shRNA) vector pGIPZ by incubating the dried plasmids in distilled water for 30 minutes prior to either 1 μl or 5 μL being added directly to

100 μL Top10 cells. The cells were incubated on ice for 5 minutes before being plated on LB plates containing 50 μg/mL carbenicillin. psPAX2, pMD2.G, and pGIPZ vectors were generous gifts from Dr. Calley Hirsch (MD Anderson Cancer Center). Alternately, the shRNA constructs targeting the Src family members were obtained directly from Open Biosystems in competent cells (Table 3.5). These cells were plated directly onto LB plates with 100 μg/mL carbenicillin and grown overnight.

3.4.3. Plasmid preparations

Three mL of LB containing 50 μg/mL carbenicillin were inoculated with a single colony from the LB plates grown overnight. These starter cultures were allowed to grow in a 37°C shaking incubator for approximately eight hours before being diluted 1:400 in 200 mL LB containing carbenicillin. Cultures were grown overnight and centrifuged at 6000 x g for 15 minutes at 4°C. Plasmids were subsequently isolated using the Qiagen Endofree Plasmid Maxi Kit as per the manufacturer's instructions.

3.4.4. Viral packaging

Briefly, transfection mixes were made containing 1.5 μg of shRNA vector, 1 μg psPAX2 and 0.5 μg pMD2.G. To create multiple knockdown cell lines cells were co-transfected with 1.5 μg of each shRNA vector. Vectors were mixed with 36 μL of Lipofectamine and incubated for 15 minutes prior to being mixed into OPTI-MEM serum free media. The entire mix was added to a 10 cm plate of 50% confluent HEK 293T cells grown in DMEM containing 10% FCS. After 5 hours, the media was changed to DMEM containing 20% FCS without P/S and cells grown under regular conditions for 48 hours. All culture media was collected and replaced with fresh DMEM containing 20% FCS. The collected media was kept at 4°C overnight. Media containing virus was harvested again at 72 hours post transfection and added to the 48 hour harvest. The harvested media was centrifuged at 1300 x g for 20 minutes prior to the supernatant being aliquoted into small volumes and kept at -80°C.

3.4.5. Transduction and selection of target cell lines

Cells were seeded so that they were approximately 50% confluent at the time of transduction. Twenty four hours after seeding, the cellular media was removed, the cells

washed with PBS and the viral supernatant added directly to the target cells in the presence of 8 μg/mL hexadimethrine bromide. After six hours, additional DMEM containing 10% FCS and P/S was added. Twenty-four hours following transduction the cells were split into larger plates and grown under normal conditions for 48 hours after which fresh media containing puromycin was added. The initial puromycin concentration depended upon cell line: 10 μg/mL for HT29 was added, 5 μg/mL for SW480 cells, 1 μg/mL for HCT116, and 2 μg/mL for HepG2 cells. Once cells had become sufficiently confluent, higher puromycin concentrations of up to 50 μg/mL were used. Media was changed every three or four days to contain fresh puromycin until no untransduced cells remained in culture, as determined using a fluorescent microscope.

3.5. RNA Isolation from Cultured Cells

RNA was isolated from confluent cultured cells according to the guanidinium thiocyanate protocol (Chomczynski and Sacchi, 1987). Following PBS wash, cell lysates were harvested in Solution D buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate ($Na_3C_6H_5O_7$), 0.5% (%w/v) N-lauroyl sarcosine) using a rubber policeman. Sodium acetate (NaOAc, 2 M), pH 4.0 was added and the samples vortexed prior to and following the addition of water saturated phenol and cloroform:isoamyl alcohol (at a 49:1 ratio). Samples were incubated on ice for 15 minutes, during which they were vortexed every 5 minutes, and the samples centrifuged at 12 000 x g for 5 minutes at room temperature. Following the transfer of the aqueous layer to a clean microfuge tube, an amount of isopropanol equal to 75% of the aqueous layer volume was added and the sample vortexed prior to being incubated at -20°C overnight. Samples were centrifuged at 12 000 x g for 15 minutes, and the pellet dried and resuspended in Solution D containing β-mercaptoethanol. Cold 95% ethanol was added and the sample incubated at -20°C for a minimum of 1 hour before the samples were centrifuged at 12 000 x g for 20 minutes at room temperature. The resulting pellet was washed once with 70% ethanol before being dried and resuspended in RNA Storage Buffer (0.1 M EDTA in water containing 0.1% (v/v) diethyl pyrocarbonate). The RNA concentration of each sample was determined by measuring the absorbance at 260 nm (A_{260}). Only samples with an A_{260}:A_{280} ratio of more than 1.6 were used.

3.6. Northern Blot

3.6.1. Northern Blot electrophoresis and gel transfer

A 1% agarose gel was made in MOPS running buffer (0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA) containing 6% (v/v) formaldehyde and poured into an RNase free electrophoresis apparatus. Samples were prepared in Northern Juice (50.7% (v/v) formamide, 11.3% (v/v) 10X MOPS, 6.8% (v/v) formaldehyde, 5.6% (v/v) glycerol in diethyl pyrocarbonate treated H_2O containing 0.1 mM EDTA) to contain 12 μg of mRNA. Each sample was incubated at 65°C for 5 minutes prior to the addition of 6 x DNA gel loading buffer containing ethidium bromide. The gel was electrophoresed in 1 x MOPS running buffer. Following electrophoresis, the gel was incubated in 50 mM sodium hydroxide (NaOH), washed in dH_2O, neutralized in 100 mM Tris, pH 8.0, and washed again in dH_2O for 20 minutes each. RNA was transferred overnight in 50 mM sodium phosphate solution, pH 7.0 onto a GeneScreen Plus hybridization transfer membrane. Following transfer, the membrane was cross-linked using a UV Stratalinker 2400.

3.6.2. Northern Blot probes

Probes used for Northern Blotting were generated by using endonuclease restriction enzymes to create DNA fragments, as described in Molecular Cloning: A Laboratory Manual (Sambrook *et al*., 1989). The probes were subsequently extracted by gel extraction using the QIAquick Gel Extraction Kit. The Src probe was generated by digesting the Src(wt) Y530 pcDNA 3.1 plasmid with *Kpn*I and *Nco*I. A probe to detect c-Yes was created by digesting the Yes EST with *Bam*HI and *Hinc*III to produce a 560 base pair (bp) cDNA fragment. Probes to detect Fyn, Lyn, and Lck were also generated by digesting their corresponding EST with restriction enzymes: the Fyn EST was digested with *Kpn*I to create a 431 bp fragment, the Lyn EST was digested with *Sac*I and *Hind*III, creating a 523 bp fragment, and the Lck EST was digested with *Rsr*II and *Nco*I, which resulted in a 500 bp probe being produced. Probes were labelled with [α-^{32}P] using Ready-To-Go DNA Labeling Beads
(-dCTP) according to manufacturer's instructions, and unincorporated nucleotides were removed using the QIAquick Nucleotide Removal Kit, following the manufacturer's protocol.

3.6.3. Northern Blot hybridization and detection

Membranes were pre-hybridized in ExpressHyb hybridization solution for 1 hour at 65° in a Micro Hybridization Incubator Model 2000 prior to the addition of the probe. Following the addition of the [α-^{32}P]-labelled probe, the hybridization reaction was allowed to proceed overnight at 65°C before the membrane was washed with a series of three 20 minute washes with decreasing concentrations of SSC buffer: Wash Buffer 1 (0.3 M sodium chloride (NaCl), 30 mM sodium citrate, 1% sodium dodecyl sulfate (SDS), Wash Buffer 2 (75 mM NaCl, 7.5 mM sodium citrate, 1% SDS), and Wash Buffer 3 (30 mM NaCl, 3 mM sodium citrate, 1% SDS). Following washing, the membrane was wrapped in saran wrap and exposed to a Molecular Imager FX Imaging Screen K before the image was viewed using a Molecular Imager FX phosphoimager. The membrane was also generally used to expose BioMAX XAR film in the presence of an intensifier screen.

3.7. Protein Isolation from Cultured Cells and Protein Concentration Determination

3.7.1. In 2X sample buffer

Cells were grown in tissue culture plates or dishes. Once they had grown to the desired confluency or for the desired time the plates were washed once with PBS and 2 x SDS Sample Buffer (10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 65 mM Tris-HCl (pH 7.0), and 0.05% (w/v) bromophenol blue) was added directly to the plate. To examine SFK expression in panels of cell lines, each cell line was grown until it just reached confluency, in order to maintain consistency. Protein was harvested by scraping and the samples were boiled and resuspended further by running through a 27 ½ gauge needle several times before protein concentration was determined. Samples were stored at -80°C.

3.7.2. In RIPA buffer

Cells were grown in tissue culture plates or dishes until either the desired confluency or time was reached. For kinase assays, all cell lines were grown until they were near confluency prior to harvesting. Cells were washed once with cold PBS and RIPA buffer (150 nM NaCl, 25 mM Tris-HCl pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA and 1 mM fresh dithiothreitol (DTT)) added directly to the plate. Cells were harvested by scraping and samples were kept at -80°C until use.

3.7.3. Lowry protein assay

The protein concentration of all samples was determined using the commercially available Micro Lowry Total Protein Kit according to the manufacturer's instructions.

3.8. Western Blot

3.8.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out following the method of Laemmli (1970). In general, the western gels used were a 5% acrylamide stacking gel (29.2% acrylamide:0.8% bis acrylamide, 130 mM Tris-HCl, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate, 0.04% (w/v) TEMED) and a 10% SDS-polyacrylamide resolving gel (10% acrylamide:bis acrylamide (29.2%:0.8%), 375 mM Tris-HCl pH 8.8, 0.1% (v/v) SDS, 0.1% (w/v) ammonium persulfate and 0.04% (w/v) N,N,N',N'-tetramethylethylenediamine (TEMED). Protein samples were boiled for 5 minutes and an equal concentration of protein loaded into the gel; in general, 30 μg of protein was used. Electrophoresis was carried out in SDS Gel Running Buffer (25 mM Tris-HCl, 200 mM glycine, 0.1% (w/v) SDS). The resulting gel was soaked for 15 minutes in Transfer Buffer (48 mM Tris, 39 mM glycine, 20 % methanol, 0.0375% SDS) and transferred to a BioTrace NT nitrocellulose blotting membrane using an OWL semi-dry transfer apparatus.

3.8.2. Western Blot procedure

Membranes were rinsed in TBST (10 mM Tris, 15 mM NaCl, 0.5% (v/v) TWEEN-20) and blocked in 5% (w/v) fat-free skim milk powder in TBST for a minimum of 1 hour at room temperature to overnight at 4°C before being incubated in primary antibody diluted in blocking solution. Following four washes with TBST, membranes were incubated in horseradish peroxidase (HRP) conjugated secondary antibody again diluted in blocking solution for 1 hour at room temperature (Towbin *et al*., 1979). Following a second set of four washes in TBST, membranes were incubated in Western Lightning chemiluminescent reagent for 1 minute. The membrane was subsequently exposed to X-OMAT Blue film for varying lengths of time and the film developed. Antibodies could subsequently be stripped from the membrane by incubating it in Stripping Buffer (62.5 mM Tris, 2% (v/v) SDS, 0.7% (v/v) β-mercaptoethanol), allowing the membrane to be blotted with different antibodies, if desired. Antibodies used in these studies may be found in Table 3.4.

3.8.3. Analysis of Western Blots with Li-Cor technology

Western blots developed using the Li-Cor system were carried out as above, except that membranes were washed in PBS or PBST (PBS with Tween 20) instead of TBST, and were blocked in Li-Cor Blocking Buffer. Antibodies were also diluted in Li-Cor Blocking Buffer containing 0.05% Tween 20. Secondary antibodies conjugated to infrared dyes were used, allowing the signal to be detected using the Li-Cor Odyssey Infrared Imaging System. Band intensities were determined using the Li-Cor Odyssey software.

3.9. Immunohistochemical Staining of Lung Cancer Tissue Array

A lung cancer tissue microarray containing 50 cases in duplicate was obtained from US Biomax, Inc.. Ten adenocarcinoma cases, ten squamous carcinoma, and eight small cell carcinoma cases were represented, along with multiple other types, as well as four non-malignant normal lung tissue samples. Samples were taken from both male and female patients ranging in age from 33 to 75 years of age. The slide was sent for immunohistochemical staining for Lyn protein at the Saskatoon City Hospital, using the sc-7274 anti-Lyn antibody. In addition, the slide was counterstained with hematoxylin in order to visualize cells. Slides were examined for the percent of cells that were positive for Lyn expression and categorized as follows: no Lyn expressed (–), <25% of cells expressed Lyn (+/-), 25% to 50% of cells expressed Lyn (+), 50% to 75% of cells expressed Lyn (++), >75% of cells expressed Lyn (+++).

3.10. Tyrosine Kinase Assay

3.10.1. Immunoprecipitation

The protein concentration of cell lysates harvested in RIPA buffer was determined using a Lowry Assay and samples were made up to 1 μg/mL in RIPA buffer containing 1 x Protease Inhibitor Cocktail (PIC), 1 mM dithiothreitol (DTT), and 100 μM sodium orthovanadate (Na_3VO_4). Ten μL of antibody was added and samples were nutated overnight at 4°C. Following incubation, 20 μL of Protein G Agarose was added to each sample before being nutated for an additional 1 hour and 15 minutes at 4°C. Samples were centrifuged at 2300 x g for 1 minute at 4°C and the beads washed twice with RIPA buffer prior to being washed once with 1 X Kinase Reaction Buffer (1 mM ATP, 10 mM $MgCl_2$, 25 mM Tris and 5 mM β-

glycerophosphate) containing 2 mM DTT and 100 μM Na_3VO_4. Assays were carried out directly on immunoprecipitated proteins.

3.10.2. Kinase assay

The kinase activity of the immunoprecipitated Src family members was determined using the Chemicon Tyrosine Kinase Activity Assay kit according to the manufacturer's instructions. The kinase reaction was allowed to proceed for 45 minutes.

3.11. Cellular Proliferation Assays

3.11.1. MTS assay

Cellular Proliferation Assays were carried out using the CellTitre 96 Aq_{ueous} One Solution Cell Proliferation Assay. Briefly, HepG2 cells were seeded and reverse transfected in triplicate in 96 well plates, while the stable knockdown HT29, SW480, or HCT116 cells were trypsinized, counted using a Coulter Counter ZM and equivalent cell numbers seeded in each well. Identical plates were seeded for each 24 hour time point (up to 120 hours), and cells allowed to grow under normal conditions. For the assays testing the effects of SFK inhibitors on proliferation, the media was replaced after 24 hours with media containing the inhibitors or the control DMSO at concentrations of 2 μM, 10 μM, or 30 μM, or the control DMSO at the corresponding volumes. To asses the number of live cells present in each well, 20 μL of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was added to each well using a multichannel pipettor and allowed to incubate for 1 hour at 37°C and 5% CO_2 before the absorbance was read at 490 nm using a microplate reader. Background was corrected for by including wells containing media without cells. To determine cellular proliferation over time MTS assays were performed every 24 hours for up to 96 hours.

3.11.2. Cellular growth curve experiments

Stable shRNA-transduced HT29 cells were starved for 24 hours in DMEM containing 0.05% FCS prior to being trypsinized, counted using a Coulter Counter ZM, and seeded in duplicate at 1.0×10^5 cells per 35 mm well (9.6 cm^2) in media containing 10% FCS. Cells were allowed to grow under normal conditions and were trypsinized and counted in PBS every 24

hours for five days using the Coulter Counter ZM. Growth curves of the HCT116 and SW480 knockdown cells were also determined; however, 1.0 x 10^5 HCT116 and 2.0 x 10^5 SW480 cells were seeded initially.

3.12. Adhesion Assay

Adhesion Assays were carried out in 96 well plates coated overnight with either 2.5 μg fibronectin or 2.5 μg collagen I. Following coating, wells were washed twice with PBS before being blocked with 0.5% bovine serum albumen (BSA) in PBS for 1 hour at 37°C, washed three times in PBS and chilled at 4°C for up to a week. To perform the adhesion assays, cells were trypsinized, counted using a Coulter Counter ZM and the cells seeded directly into the wells. 5.0 x 10^4 HepG2 and HCT116, 1.0 x 10^5 HT29, or 1.5 x 10^5 SW480 cells were seeded per well and incubated at 37°C for 1 hour. To assess the adhesion of the stable shRNA expressing cells, a suspension of cells in 100 μL was seeded directly into the wells. To assess the effect of SFK inhibitors on cellular adhesion, the inhibitors or DMSO were diluted to concentrations of 4 μM, 20 μM, or 60 μM in 100 μL and 50 μL added to each well. The above numbers of cells were then seeded into the wells in 50 μL for a final inhibitor concentration of 2 μM, 10 μM, or 30 μM. Following incubation, wells were washed with PBS to remove unadherent cells from the wells before the adherent cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Following two washes with PBS, cells were stained with crystal violet (5 mg/mL in 2% ethanol) for 10 minutes. After washing with water, wells were allowed to dry before 2% SDS was added. Following a 30 minute incubation at room temperature, absorbance was read at 550 nm with a microplate reader.

3.13. Wound Healing Assay

3.13.1. Tissue culture

Confluent HT29, SW480, or HepG2 cells were trypsinized, counted using a Coulter Counter ZM, and 1.0 x 10^6 HT29, 1.5 x 10^6 SW480, or 6.0 x 10^5 HepG2 cells seeded into 35 mm wells containing a sterile etched glass slide. The cells were allowed to grow for 24 hours (HT29 and HepG2) or 48 hours (SW480) until they reached 80 to 90% confluency. Once the cells had reached this confluency, the media was changed to starvation DMEM containing 0.5% FCS and the cells allowed to grow for a further 24 hours. To assess migration, a sterile 10 μL

pipette tip was used to score three separate lines into the monolayer, the well washed at least twice with PBS to remove debris, and DMEM containing 10% FCS added to the well. The plates were placed on a Nikon Eclipse TE300 microscope and 0 hour photographs were taken using a Nikon Coolpix 990 digital camera at distinct locations under 10x magnification. Cells were placed under normal growth conditions and allowed to grow for 24 or 48 hours before photographs were taken at the same locations as the 0 hour photographs. Six photographs, two from each wound, were taken for each well. In order to determine the effect of SFK inhibitors on cellular migration wound healing assays were carried out exactly as described, except that following wounding the complete media added to the cells contained the indicated inhibitors at the given concentration.

3.13.1. Quantification of migration

In order to quantify migration, the width of the wound was measured at the same locations of the slide at both 0 hours and 24 or 48 hours. Image J software was used to measure the width of the wound at three locations for each photograph, using the slide etchings as a guide. Subsequently, the distance migrated in pixel width was calculated, and corrected to the pixel width migrated by the control cells.

3.14. Colony Forming Assay

3.14.1. Seeding

Bottom agarose layers were formed by plating 0.6 % agarose made up in 2 X DMEM plus 20 % FCS and allowing the agarose to set. Cells were counted using the Coulter Counter ZM and 5000 HT29, HepG2, or HCT116 cells or 10 000 SW480 cells were resuspended in 0.3 % agarose containing 10 % FCS. This cell suspension was layered over the bottom agarose layer, allowed to set, and the cells placed at 37°C plus 5 % CO_2 for 14 days for HepG2 cells to 21 days for HT29. Every three to four days fresh 0.3 % agarose in DMEM was layered over the wells. To assess SFK Inhibitor effects on colony forming ability, cells were seeded in the presence of 2 μM, 10 μM, or 30 μM of each inhibitor. In these assays, the inhibitors were also present at the indicated concentrations every time additional agarose was overlayed throughout the course of the assay.

3.14.2. Counting colonies formed

Colonies were counted manually, using a grid. Four random areas of each well were counted under a microscope and the average number of colonies determined for each well. All experiments were carried out in duplicate.

3.15. Statistical Analysis

Data is presented as the mean ± standard deviation. One-way analysis of variance was performed on all data using GraphPad Prism, version 5.0d for Mac OS X (GraphPad Software, San Diego, CA, www.graphpad.com). Dunnett's multiple comparisons post test was performed to compare the mean of each sample to the mean of a control sample; either cells treated with the corresponding volume of DMSO for experiments looking at the effects of Src kinase inhibitor treatments, or those transduced with the empty shRNA control vector (pGIPZ), or transfected with a scrambled control siRNA, for experiments examining the effects of decreased Src, Fyn, or Yes expression. *P* values of less than 0.05 were considered to be statistically significant.

4. RESULTS

4.1. SRC FAMILY KINASE EXPRESSION AND ACTIVITY IN A PANEL OF CELL LINES

4.1.1. Src family kinase mRNA expression in a panel of colon cancer cell lines

In order to investigate the mRNA expression of the SFKs in a panel of colon cancer cell lines, mRNA was isolated from each cell line and Northern Blots carried out. In the nine colon cancer cell lines initially examined by Dr. Calley Hirsch, five SFKs (Src, Yes, Fyn, Lyn, and Lck) were found to be expressed at the mRNA level in at least a subset of the cell lines investigated, while Blk, Fgr, and Hck showed no mRNA expression (Figure 4.1). Furthermore, it was found that the different colon cancer cell lines examined each expressed only a subset of the SFKs, and that these combinations differed between cell lines. In addition, the SFKs were expressed at different levels in the different cell lines. To expand on this finding, a further seven colon cancer cell lines were examined for SFK mRNA expression, and were similarly found to express different combinations of SFKs.

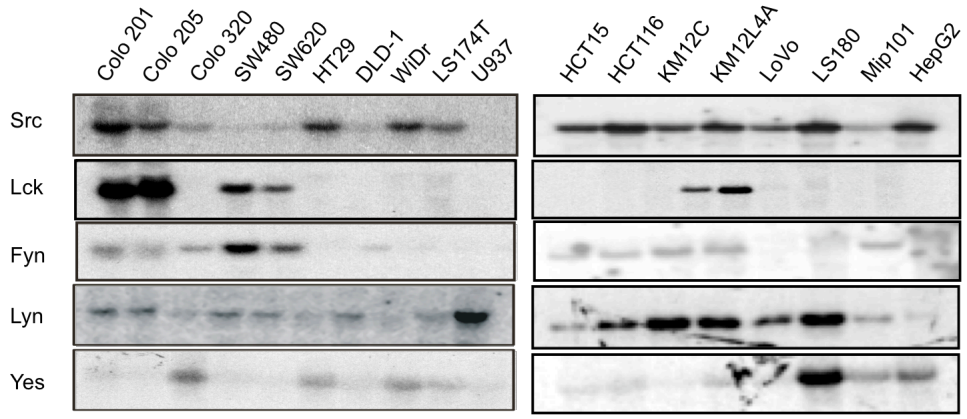


Figure 4.1. mRNA expression of the Src family kinases in different cancer cell lines. Total mRNA was harvested from confluent plates of sixteen colon cancer cell lines, the HepG2 hepatocellular cell line, and the U937 myeloid cell line. The mRNA expression of the Src family members was examined by Northern Blot using probes specific for Src, Lck, Fyn, Lyn, or Yes. Blk, Fgr, and Hck were not detected.

4.1.2. Src family kinase protein expression in a panel of colon, breast and lung cancer cell lines

Upon determining the levels of SFK mRNA expression in a panel of colon cancer cell lines, it was of interest to examine the protein expression of these kinases as well. Therefore, the protein expression of the SFKs was investigated in the same panel of colon cancer cell lines, as well as in a panel of five breast cancer cell lines, six lung cancer cell lines and two normal lung cell lines, the hepatocellular carcinoma cell line HepG2, and the myeloid cell line U937. At confluence, when the cells covered the maximum percentage of the tissue culture plate attainable by each given cell line, each of the cell lines was harvested for protein, and Western Blots carried out to determine the level of protein expression of each of the SFKs. The level of protein expression correlated to what was observed at the mRNA level in most cell lines, and the Western Blots similarly demonstrated that Src, Yes, Fyn, Lck and Lyn proteins are expressed by the various cell lines at different levels and in different combinations (Figure 4.2). For example, HT29 cells express both Src and Yes at relatively high levels, and Fyn at a lower level, when compared to other cell lines. In contrast, SW480 cells express Src and Yes at lower levels and Lck and Fyn at relatively high levels. The majority of the cell lines examined also expressed Lyn. From these observations, the colon cancer cell lines HT29, SW480, and HCT116 were chosen for further study, as they are representative of the different combinations of SFKs expressed in the panel of cell lines. The hepatocellular carcinoma cell line HepG2 was also included in further studies as it expresses high levels of Src and has been used extensively in our laboratory.

4.1.3. Src family kinase activity in selected cancer cell lines

Following the observation that a given cell line may express multiple SFKs at the mRNA and protein level, the kinase activity of the SFKs was examined in selected cell lines using a commercially available Tyrosine Kinase Activity Assay kit (Millipore) that can be used to assess the kinase activity of specifically immunoprecipitated proteins. Following immunoprecipitation, the tyrosine kinases are incubated with a synthetic peptide, and the level of the resulting phosphorylation measured colourimetrically using an HRP-conjugated phosphotyrosine antibody. As the SFKs can be immunoprecipated using specific antibodies, the kinase activity of each of the individual SFKs can be assessed. Although multiple SFKs

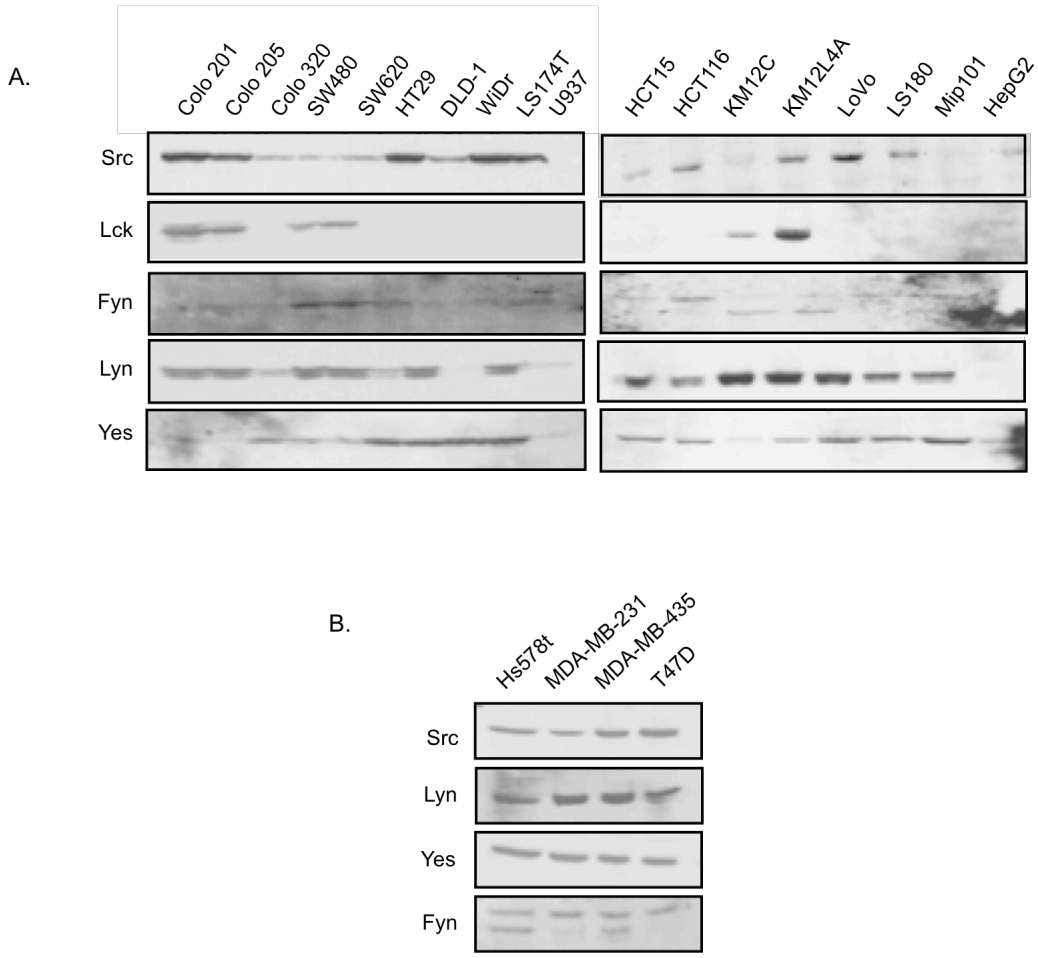


Figure 4.2. Protein expression of the Src family kinases in different cell lines. Protein was harvested from confluent cells and Western Blots carried out using Src, Lck, Fyn, Lyn, or Yes specific antibodies to detect Src family member expression in **A.** Sixteen colon cancer cell lines, the HepG2 hepatocellular cell line, and the U937 myeloid cell line, and **B**. Four breast cancer cell lines.

were found to be expressed at the protein level in a given cell line, not all of the SFKs expressed exhibited kinase activity (Figure 4.3). For instance, although Lyn is expressed in HCT116 cells, Lyn kinase activity above the level observed in control samples that were not incubated with immunoprecipitated protein was not detected. In general, Src was found to have the highest level of kinase activity, although Yes activity was also detected in HT29 cells and, to a lesser extent, HepG2 cells. The relative Src kinase activity also differed between cell lines, with HT29 having the highest activity. In SW480 cells, only Src and Fyn were found to have kinase activity. As may be expected from the lower level of Src protein expressed in SW480

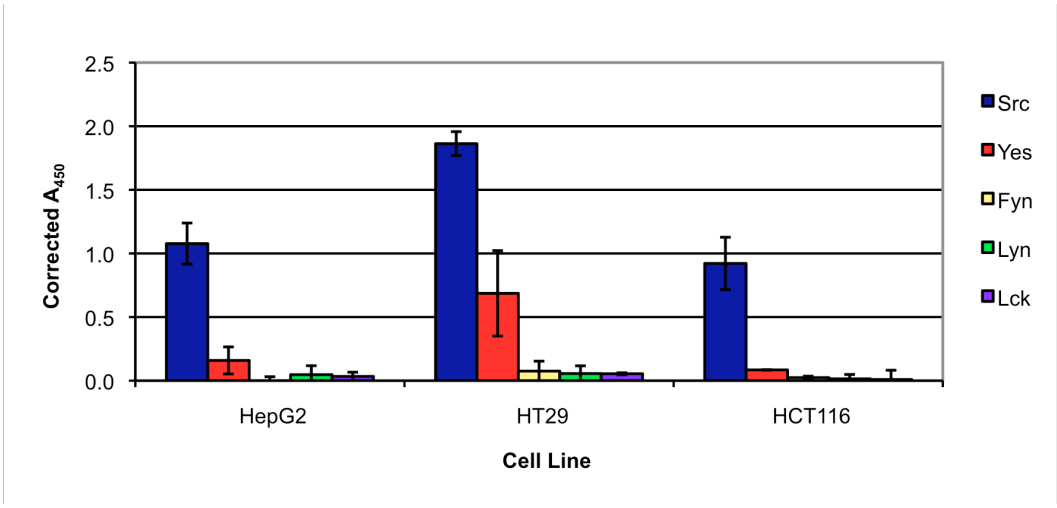


Figure 4.3. Src family member kinase activity in HepG2, HT29 and HCT116 cells. Src family members were immunoprecipitated from cell lysates of each cell line using antibodies specific for each of the five Src family members expressed, as indicated, and the kinase activity of each detected using a non-radioactive kinase assay kit. Error bars are representative of the standard deviation of three independent experiments carried out in duplicate.

cells when compared to the other cell lines examined, the level of Src kinase activity in SW480 cells was relatively low. In addition to SW480 cells, the SW620 cell line, which is derived from the lymph node metastasis of SW480 cells, was also assayed for SFK kinase activity. Much like the SW480 cells, SW620 showed Src and Fyn kinase activity; however, the level of kinase activity of both of these proteins was increased in the SW620 cells (Figure 4.4).

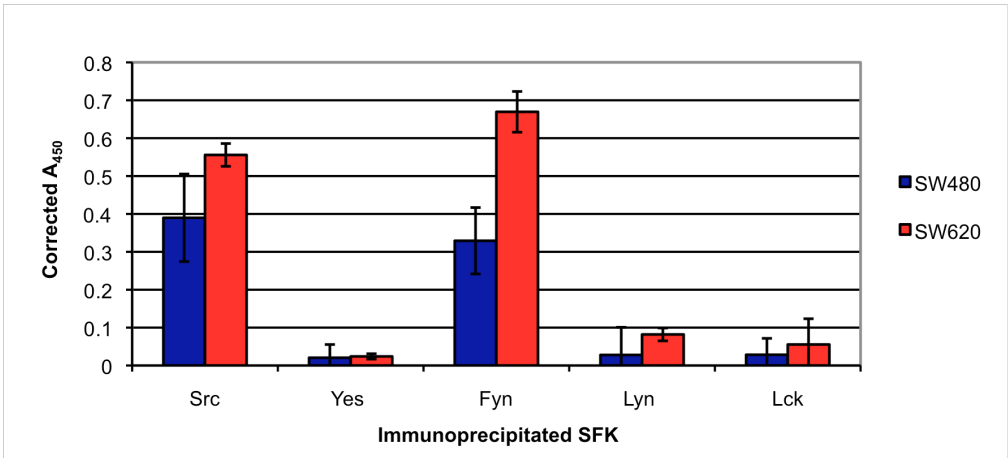


Figure 4.4. Src family member kinase activity in SW480 and SW620 colon cancer cells. Src family members were immunoprecipitated from cell lysates of each cell line using antibodies specific for each of the five Src family members expressed, as indicated, and the kinase activity of each detected using a non-radioactive kinase assay kit. Error bars are representative of the standard deviation of four (SW480) or two (SW620) independent experiments carried out in duplicate.

4.1.4. Src family kinase protein expression and kinase activity in a panel of lung cancer cell lines

In addition to colon and breast cancers, SFKs have also been found to be overexpressed and activated in lung cancer (Mazurenko *et al*., 1992; Masaki *et al*., 2003; Zhang *et al*., 2007). Therefore, in addition to the colon cancer and breast cancer cell lines previously examined, a panel of normal lung and lung cancer cell lines was examined for both SFK protein expression and Src and Lyn kinase activity. In agreement with previous findings in the other cell lines examined, the lung cell lines all expressed multiple SFKs at different levels; the majority of the lung cell lines, including the two normal lung lines CCD-16Lu and CCD-19Lu, were found to express Src, Yes and Fyn (Figure 4.5 A). Src was expressed at similar levels in all but two of the cell lines, and all of the cell lines showed similar levels of Yes protein expression. Fyn was also detected in all but one of the lung lines. Similar to what was observed in the panel of colon cancer cell lines, where Lck was expressed in only a small subset of the cell lines, Lck was detected at very low levels in only the NCI-H727 lung cancer cell line. Although the majority of the colon and breast cancer cell lines examined were found to express Lyn, this protein was detected in only four of the eight lung lines. Therefore, to determine if Src and/or Lyn kinase were active in these lung cell lines, the same kinase assay previously used to detect SFK kinase activity in HT29, HCT116, SW480, SW680 and HepG2 cells was employed. Src activity was detected in all of the cell lines examined (Figure 4.5 B), while the NCI-H2009 and HCC-827 cell lines were also observed to have Lyn activity. No significant Lyn activity was detected in the H23 and H727 cell lines, which correlates with the lower levels of Lyn protein expressed in these cell lines, when compared to NCI-H2009 and HCC-827 cells.

4.1.5. Lyn expression in a lung cancer tissue array

Lyn has previously been found to be expressed in lung tissue (Holtrich *et al*., 1991; Pertel *et al*., 2006; Zhao *et al*., 2006); however, the involvement of Lyn in lung cancers has not been studied extensively. Therefore, as Lyn was found to have kinase activity in the lung cancer cell lines in which it was expressed, it was of interest to examine Lyn expression in samples of lung cancer tissue. To this end, a lung cancer tissue microarray was obtained from US Biomax that contained duplicate samples of ten adenocarcinoma and ten squamous cell carcinomas, both generally from stage II or III cancers, as well eight small cell lung cancer cases, and other

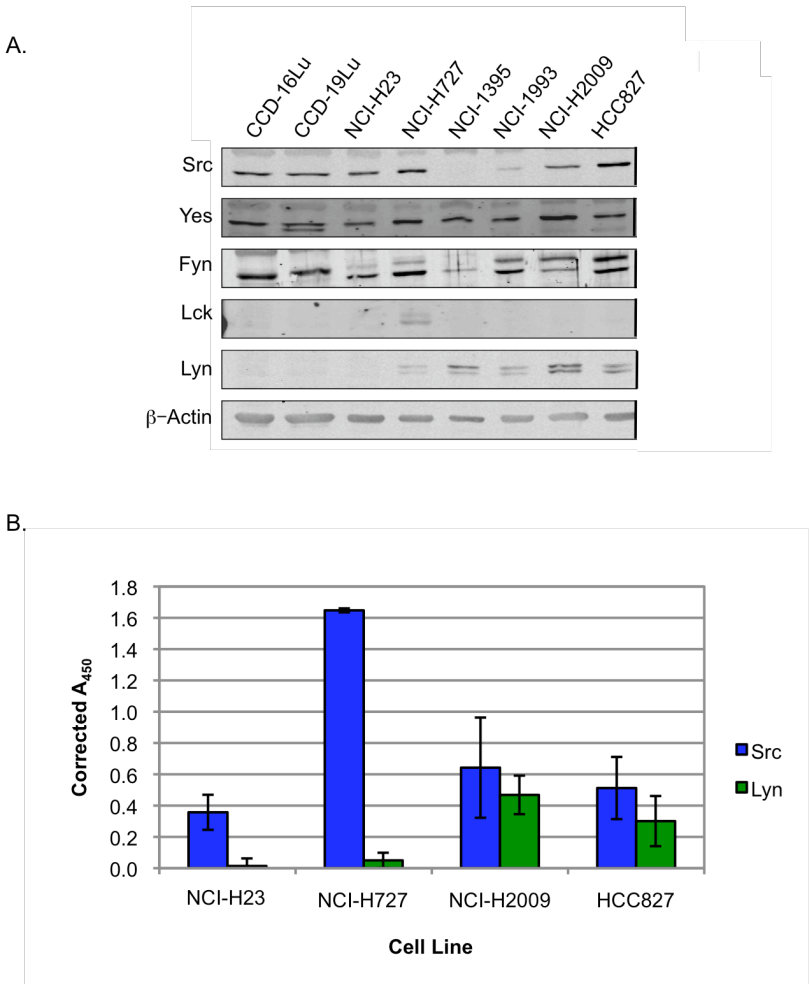


Figure 4.5. Src family member protein expression and Src and Lyn kinase activity in a panel of lung cancer cell lines. A. Confluent plates of normal lung and lung cancer cell lines were harvested in 2 X Sample Buffer and 30 μg of protein electrophoresed in an SDS-PAGE gel. Western blots were carried out using antibodies against Src, Yes, Fyn, Lck, Lyn and β-Actin, as indicated. **B.** Src or Lyn were immunoprecipitated from total protein harvested from the indicated cell lines and the kinase activity of these proteins determined using the Chemicon Tyrosine Kinase Assay kit. Data presented is the average of at least two independent experiments carried out in duplicate.

lung cancers. Four non-malignant normal lung specimens were also included. Following DAB-immunohistochemical staining using a Lyn specific antibody, which is visualized as a brown stain, and counterstaining with hematoxylin, which non-specifically stains cells blue and allows the visualization of cells, cases were examined for Lyn expression in order to determine if any patterns of expression were notable. When examined, the majority of cases showed some Lyn expression, although generally at low levels, and often in fewer than 50% of the cells

within the sample. However, some histological types of lung cancer showed higher levels of Lyn expression, both in terms of the number of cells positive for Lyn expression, as well as in Lyn protein levels. In particular, the large cell carcinoma and atypical carcinoid samples showed high Lyn expression, with many of the cells in these cases expressing Lyn. A high percentage of cells in the squamous cell carcinoma samples also expressed relatively high levels of Lyn. In contrast, the majority of the adenocarcinoma samples showed no or very low levels of Lyn expression. Within these cases, where the stage of cancer was known, no correlation between Lyn expression and the stage of cancer was observed. The number of cases of each lung cancer pathology with a certain percentage of cells expressing Lyn is given in Table 4.1, while representative slides of adenocarcinoma, squamous cell carcinoma, small cell and large cell lung carcinomas, as well as papillary adenocarcinomas, alveolar cell carcinoma, papillary adenocarcinomas, atypical carcinoid, and non-malignant normal lung tissue are shown in Figure 4.6.

Table 4.1. The number of cases of a lung cancer tissue array that show positive staining for Lyn protein expression. Following immunohistochemical staining with a Lyn specific antibody, each case of a lung cancer tissue array obtained from US Biomax was examined and the percent of cells within each sample that were positive for Lyn expression determined. The number of cases within each lung cancer type that were positive for Lyn expression in the indicated percentage of cells is given.

Lung cancer type (total number of cases)	Number of cases with the indicated percent of cells positive for Lyn expression				
	0%	0-25%	25-50%	50-75%	75-100%
Non-malignant lung (4)	0	2	1	1	0
Adenocarcinoma (10)	6	1	1	1	1
Squamous (10)	0	1	3	4	2
Small cell (8)	1	3	3	1	0
Large cell (4)	0	0	0	1	3
Atypical (4)	0	0	0	0	4
Papillary (2)	0	0	1	0	1
Alveolar (2)	0	0	0	1	1
Adenosquamous (2)	0	0	0	2	0
Mucinous alveolar (1)	0	0	0	0	1

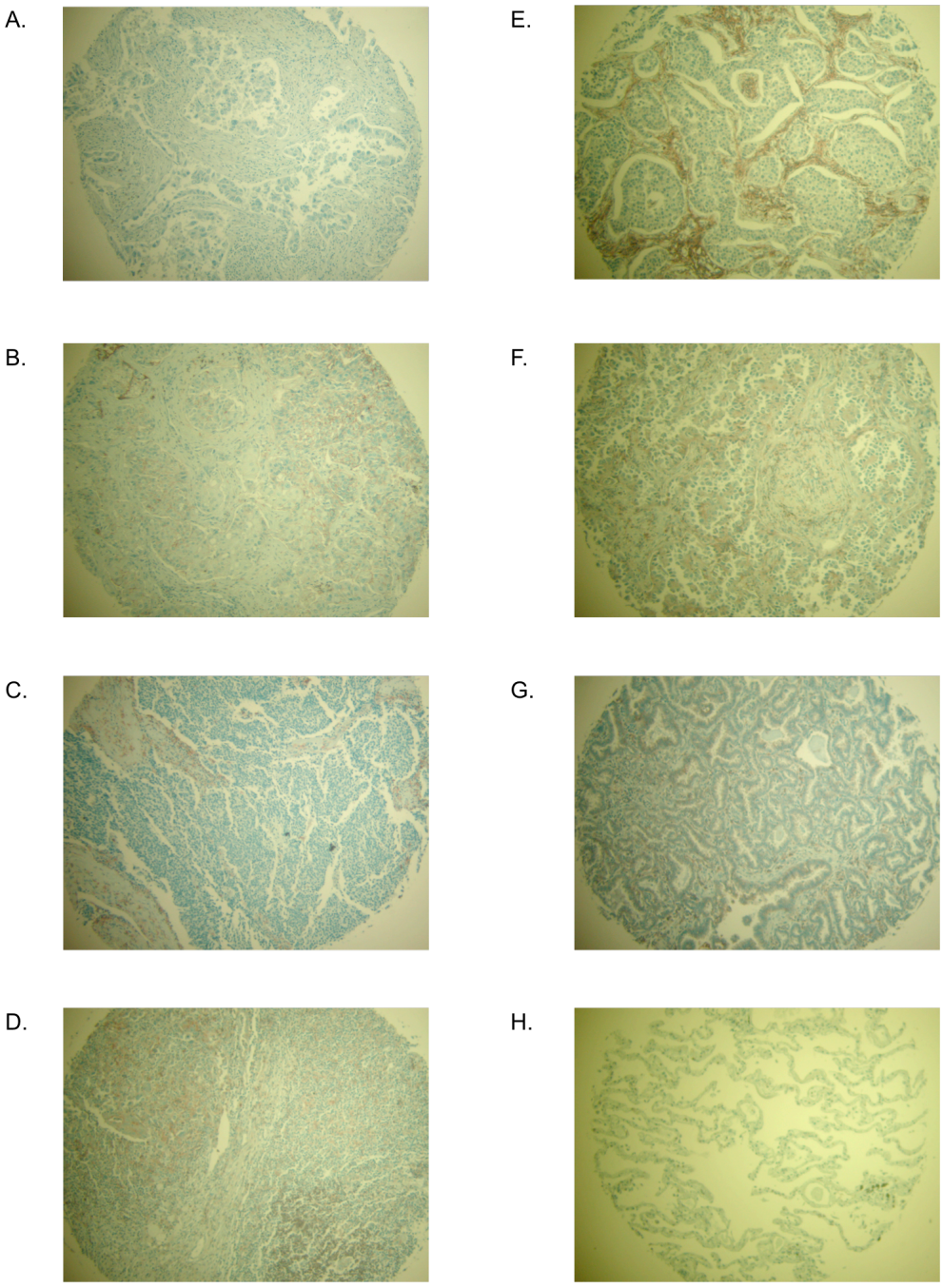


Figure 4.6. Lyn expression in a panel of lung cancer tissue specimens. Immunohistochemistry was carried out on a lung cancer tissue microarray using a Lyn specific antibody (brown) and a hematoxylin counter stain (blue). Representative samples of each lung cancer type are given as follows: A. Adenocarcinoma, B. Squamous cell carcinoma, C. Small Cell, D. Large Cell, E. Atypical, F. Papillary Adenocarcinoma, G. Alveolar, and H. Normal lung tissue.

4.1.6. Summary

When the expression and kinase activity of the individual SFKs was examined in a panel of cancer cell lines it was found that although each cell line expressed multiple SFKs, the particular subset of SFKs expressed differed between cell lines. In addition, the level of protein expression of each SFK varied between cell lines. When selected cell lines were examined for SFK kinase activity it was found that Src itself exhibited kinase activity in all of the cell lines examined. Yes or Fyn activity was also detected in selected colon cancer or hepatocellular carcinoma cell lines, while Lyn activity was detected in both of the lung cancer cell lines that expressed high levels of Lyn protein. When Lyn expression was examined in lung cancer tissue present on a tissue microarray it was found that many of the samples expressed some level of Lyn protein, although in most cases weakly.

4.2. OPTIMIZATION OF SILENCING INDIVIDUAL SRC FAMILY KINASES

4.2.1. Identification of individual siRNA sequences that effectively silence single Src family kinases

In order to investigate the contributions of the individual SFKs to different cellular phenotypes, it was necessary to be able to decrease the expression of each SFK without affecting the activity or expression of the other family members. In order to do this, individual siRNA sequences able to target each of the ubiquitously expressed SFKs, Src, Fyn, and Yes, were identified. Initially, pools of four chemically synthesized siRNAs targeting each individual SFK, known as SMART Pools, were obtained from Dharmacon. Following initial optimization using this pool of four sequences, the most effective individual sequence in each was identified by transfecting a cell line that expressed the given protein at high levels with the individual sequences and monitoring for decreases in protein expression 48 hours following transfection. The siRNA sequence that was most effective in decreasing the protein expression of Src, Fyn, or Yes was then used in further experiments in HepG2 cells (Figure 4.7).

4.2.2. Time courses of siRNA silencing

As siRNA silencing using chemically synthesized siRNAs is a transient event, it was necessary to demonstrate that the protein expression of the target SFKs remained at low levels throughout the length of our experiments. Therefore, time courses monitoring the siRNA-

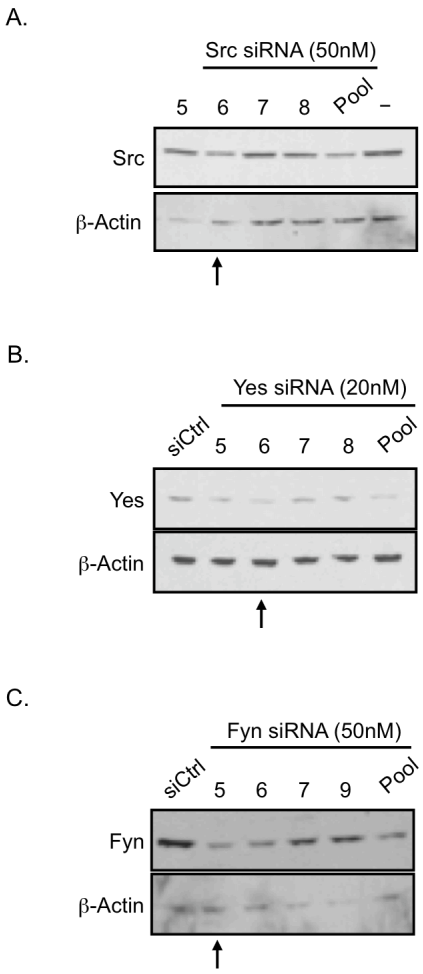


Figure 4.7. Western Blots showing the relative efficiencies of individual siRNA sequences to silence the Src family kinases. Representative blots are given for **A.** Src siRNA in HepG2 cells, **B.** Yes siRNA in HT29 cells, and **C.** Fyn siRNA in SW480 cells. Cells were transfected with the indicated concentrations of the four numbered individual siRNA sequences, the SMARTpool siRNA (Pool), the scrambled control siRNA (siCtrl), or were left untransfected (−). Western blots with the indicated antibodies, or β-actin as a loading control were performed using lysates harvested 48 hours following transfection. For each Src family member the single sequence that resulted in the greatest silencing was identified (indicated with arrow).

mediated knockdown of the SFKs were carried out in order to determine if SFK levels were significantly decreased for long enough to allow changes in cellular phenotypes to be detected. The length of knockdown was determined by harvesting protein samples at 24 hour intervals following transfection with siRNA and performing Western Blots to detect protein expression (Figure 4.8). It was demonstrated in HepG2 cells that Src levels were knocked down by 24

hours post-transfection, and that by 48 hours there was nearly complete knockdown, which was maintained for a minimum of six days. The level of Yes expression was not decreased to the same extent as the level of Src; however, expression also remained decreased past six days post-transfection. In addition to transfecting HepG2 cells with siRNAs targeting either Src or Yes, cells were transfected with siRNAs targeting both Src and Yes together, in order to knockdown the expression of both of these proteins within single cells. As was observed when these SFKs were silenced individually, Src levels were decreased nearly completely by 48 hours, while Yes knockdown was less significant. However, both Src and Yes protein levels were again found to be decreased for over six days (Figure 4.8). This duration should provide enough time to monitor a number of cellular phenotypes following siRNA transfection, including proliferation, adhesion, migration, and colony forming ability.

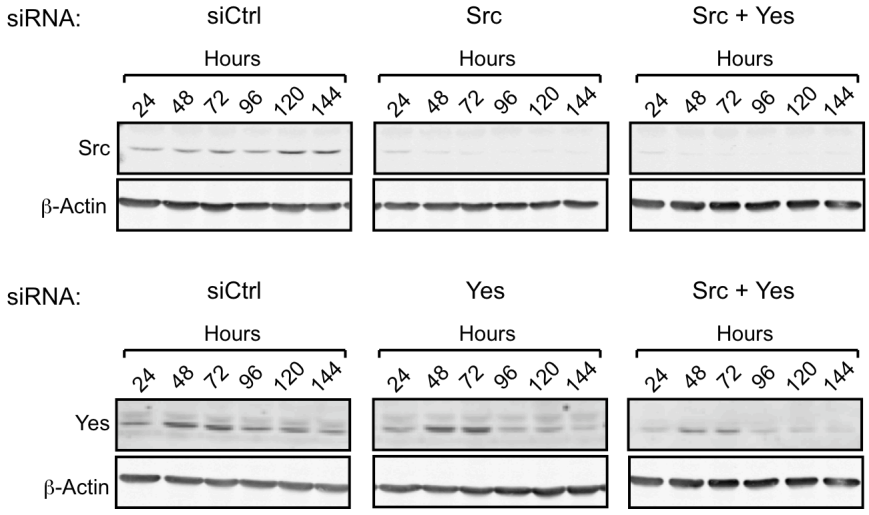


Figure 4.8. Time courses of siRNA-mediated knockdown of Src and Yes expression in HepG2 cells. HepG2 cells were seeded and reverse transfected in 35 mm wells with the indicated siRNAs, and cell lysates harvested in 2x SDS Sample Buffer every 24 hours for 144 hours. Thirty μg of the resulting lysates were then separated on an SDS-PAGE gel, and Western blots carried out using either Src or Yes specific antibodies, to determine the extent of silencing, or a β-Actin antibody, as a loading control.

4.2.3. Generation of stable cell lines with decreased Src, Fyn, or Yes expression

As siRNA silencing proved to be inconsistent in HT29, HCT116, and SW480 cells, and often did not result in a significant decrease in SFK levels, it was necessary to achieve knockdown using an alternate method. To this end, stable cell lines were created using shRNA vectors targeting individual SFKs. Pre-designed pGIPZ vectors containing shRNA sequences targeting either Src, Fyn, or Yes, were purchased directly from Open Biosystems and transfected into HEK 293T cells along with the two viral packaging vectors psPAX2 and pMD2.G. The HEK 293T cells subsequently produced lentivirus packaged with vectors containing specific shRNA sequences targeting either Src, Fyn, Yes, or the empty pGIPZ vector, as a control. Following the production of lentivirus, HT29, HCT116, and SW480 cells were transduced with the shRNA vectors. In an attempt to target multiple SFKs, cells were also transduced with combinations of lentivirus produced from HEK 293T cells that were transfected with a single shRNA vector, or with lentivirus from HEK 293T cells that were transfected with multiple shRNA vectors targeting different SFKs. Once our cell lines of interest were transduced, the cells were allowed to recover for four to six days before selection with puromycin was carried out. A puromycin kill curve was initially carried out to determine the minimum required concentration of puromycin required to kill a given cell line; however, a higher concentration was necessary in order to select for cells that contained multiple vectors. Selection for cells expressing shRNAs was carried out for several weeks, with media containing fresh puromycin changed every four days. As the puromycin selection resulted in significant cell death, cells were allowed to grow until they appeared healthy and confluent, at which point they were harvested, and Western Blots carried out in order to determine if the knockdown of the SFKs was successful (Figure 4.9 A, B, C). The percent knockdown was determined using the Odyssey software, available from Li-Cor, which measures the intensity of protein bands. After correcting for differences in the concentration of protein loaded in each well using the intensity of the β-actin band as a loading control, the level of knockdown was assessed quantitatively by comparing the intensities of protein bands from cells in which Src, Fyn, or Yes were knocked down to those of the pGIPZ control cells. Although multiple shRNA sequences targeting each of the SFKs were tested for effective knockdown, only a single sequence for each SFK was found to result in significant knockdown (data not shown). However, even the most effective sequence targeting each SFK did not result in complete

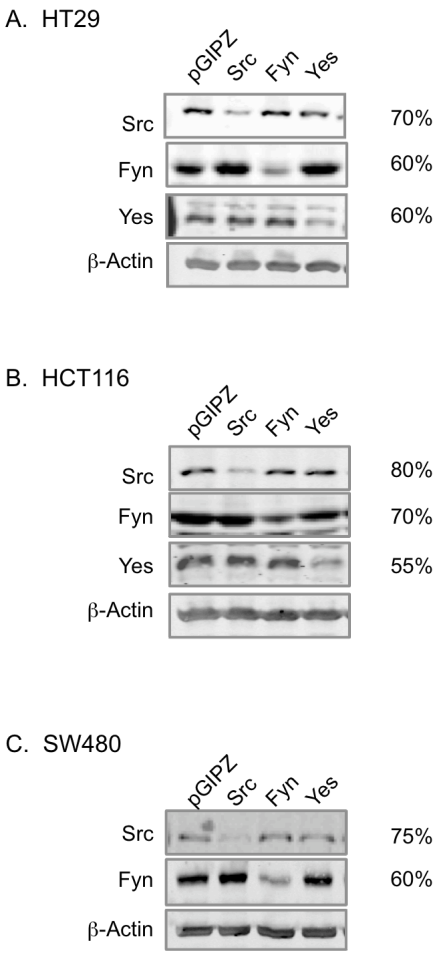


Figure 4.9. Stable cell lines with decreased Src, Fyn, or Yes expression. A. HT29, **B.** HCT116, and **C.** SW480 cells were transduced with shRNA vectors targeting Src, Yes, or Fyn. Following selection with puromycin, protein was harvested and Western blots carried out using the indicated antibodies to determine the level of protein knockdown. The level of β-actin expression was also determined for all blots as loading controls; a sample blot is shown for each cell line. At least three separate protein harvests and Western blots were carried out for each cell line, and the intensities of the protein bands measured quantitatively using the Odyssey software available from Li-Cor. The percentage by which the expression of the indicated SFKs was decreased when compared to the pGIPZ control cells is given.

knockdown of any of the SFKs (Figure 4.9). Although the level of knockdown varied between cell lines, Src levels were decreased the most significantly of the three SFKs in all of the cell lines tested, with a minimum knockdown of 70% when compared to the Src levels expressed in the control cells. Unfortunately, the decreases in Fyn and Yes expression were not as significant as that of Src in any of the cell lines, with Fyn being knocked down by 60 to 70%,

and Yes expression being decreased by approximately 60%. The level of knockdown achieved also depended upon cell line, with the greatest knockdown of all three of the SFKs occurring in HCT116 cells, while less significant SFK knockdown was observed in HT29 cells. Furthermore, as SW480 cells express very low levels of Yes protein, it was not possible to determine if Yes levels were further decreased in this cell line by the expression of a Yes-specific shRNA. SW480 cells that had been transduced with lentivirus containing the Yes specific siRNA were, however, used in subsequent experiments for the sake of completeness, and may be viewed as a further control. HepG2 cells stably expressing the shRNAs, as evaluated by decreased SFK expression, were not successfully produced (data not shown). Therefore, SFK expression was transiently decreased in HepG2 cells using chemically synthesized siRNAs in further experiments.

4.2.4. Summary

As transfection of chemically synthesized siRNAs did not result in significant or consistent enough knockdown of the SFKs in HT29, HCT116, and SW480 cells, it was necessary to move to an shRNA approach in which cells with stable knockdown of Src, Fyn, or Yes were produced. Although the transduction of HepG2 cells with shRNA vectors did not result in SFK knockdown, the transfection of siRNAs targeting Src into this cell line resulted in nearly complete knockdown of Src and this method was therefore used to decrease SFK expression in HepG2 cells in further experiments. Although the level of knockdown achieved varied between both cell line and the SFK being targeted, a decrease in protein expression of at least 55% was observed for all. Although this is less than ideal silencing, we judged this sufficient to observe if SFK knockdown results in changes in cellular phenotypes.

4.3. INVOLVEMENT OF SRC FAMILY KINASES IN CANCER CELL PHENOTYPES

Once HT29, HCT116, and SW480 cells with stably decreased Src, Fyn, or Yes expression were created, they were used to investigate the involvement of these proteins in the proliferation, adhesion, migration, and colony-forming ability of these cell lines. HepG2 cells with siRNA-mediated knockdown of the SFKs were also examined. By comparing the phenotypes of cells with decreased SFK expression to those of parental control cells and cells containing an empty control vector or scrambled siRNA control sequence it was possible to

determine which phenotypes the individual SFKs were involved in. In addition, cells in which the kinase activity of all of the SFKs was inhibited through treatment with one of four different SFK inhibitors were examined for phenotypic changes. Each inhibitor was used at three different concentrations representative of the concentrations used in the literature (2 μM, 10 μM, and 30 μM), in order to examine the effects of both low and high concentrations. By comparing the phenotypic changes resulting from SFK inhibition to those that occurred in response to the decreased expression of a single SFK it was possible to assess which SFKs in particular are involved in a given phenotype, and whether multiple SFKs must be targeted in order to cause significant effects on selected cellular phenotypes.

4.3.1. Cellular Proliferation

One cellular phenotype that Src and the other SFKs have been implicated in is cellular proliferation. In our investigations, cellular proliferation was initially determined using a commercially available MTS assay, which gives a representation of the number of metabolically active cells present in a well through the conversion of the MTS reagent to a colourimetrically detectable product. In order to assess proliferation, cells were seeded in triplicate and an MTS assay carried out every 24 hours from 0 to 72 hours, at which time the cells reached confluency. The proliferation of HepG2 cells was measured over 48 hours, as confluency was reached at this time. In the experiments examining the effects of SFK inhibition, cells were grown in the presence of the SFK inhibitors over the course of the experiment. The proliferation of cells in which Src, Fyn, or Yes levels were decreased was also examined, both by using the MTS assay, as well as by manually counting the number of cells present every 24 hours following seeding in order to create a growth curve. This allowed the effects of inhibiting the kinase activity of multiple SFKs on proliferation to be compared to those of decreased Src, Fyn, or Yes expression.

4.3.1.1. Effects of Src family kinase inhibitor treatment on cellular proliferation

In order to determine the effect of SFK inhibition on cellular proliferation, HT29, HCT116, SW480, and HepG2 cells were treated with a range of concentrations of the commercially available SFK inhibitors PP2, SU6656, SKI I and SKI II, which all inhibit multiple SFKs. It was found that not only were the four inhibitors disparate in their effects on the proliferation of

the cell lines tested, but that these effects were also cell line-specific. As expected, the ability of the SFK inhibitors to inhibit cellular proliferation was also concentration specific, with higher concentrations of the inhibitors generally having a greater effect. For example, the proliferation of HT29 cells treated with 30 μM of both PP2 and SKI I was reduced by approximately 45% and 25%, respectively, which was a greater decrease than was observed when cells were treated with either 2 μM or 10 μM of these inhibitors. Neither SU6656 nor SKI II treatment resulted in a significant effect on HT29 proliferation (Figure 4.10 A-D). When the effect of SFK inhibitor treatment on the proliferation of HCT116 was examined, it was found that the proliferation of these cells was decreased by 40% when treated with the highest concentration of PP2, 30% when treated with SU6656, and 15% when treated with SKI II. SKI I had no effect on HCT116 proliferation (Figure 4.11 A-D). In contrast, none of the SFK inhibitors tested had a significant effect on the proliferation of SW480 cells (Figure 4.12 A-D) or HepG2 cells (Figure 4.13 A-D). Although the proliferation of cells treated with DMSO presented represents the proliferation of cells treated with a volume of DMSO equivalent to that of the 10 μM inhibitor samples, three separate DMSO controls were utilized and showed no significant difference in their effect on proliferation.

4.3.1.2. Effects of decreased Src, Fyn, or Yes expression on cellular proliferation

Once the effect of SFK inhibition on proliferation was determined, the proliferation of HT29, HCT116, SW480, and HepG2 cells with decreased Src, Fyn, or Yes expression was investigated, in order to compare the effects of inhibiting multiple SFKs to those when the activity of a single SFK was decreased. Initially, the MTS assay was again used to measure proliferation. However, due to the difficulty in ensuring that the same number of cells was seeded in every experiment, and the small number of cells seeded, the results obtained using this assay were quite variable, with a large statistical error between replicates. When the fold growth of the cells was determined, which corrects for the number of cells seeded per well, the data obtained from all replicates was less variable. Although no effect on proliferation was observed in HT29 cells when using the MTS assay (Figure 4.14 A), HT29 cells expressing lower levels of both Src and Yes were qualitatively observed to reach confluency more slowly than the cells containing the control pGIPZ vector, those with decreased Fyn expression, or the parental HT29 cells. Growth curves were therefore also created in order to monitor cellular

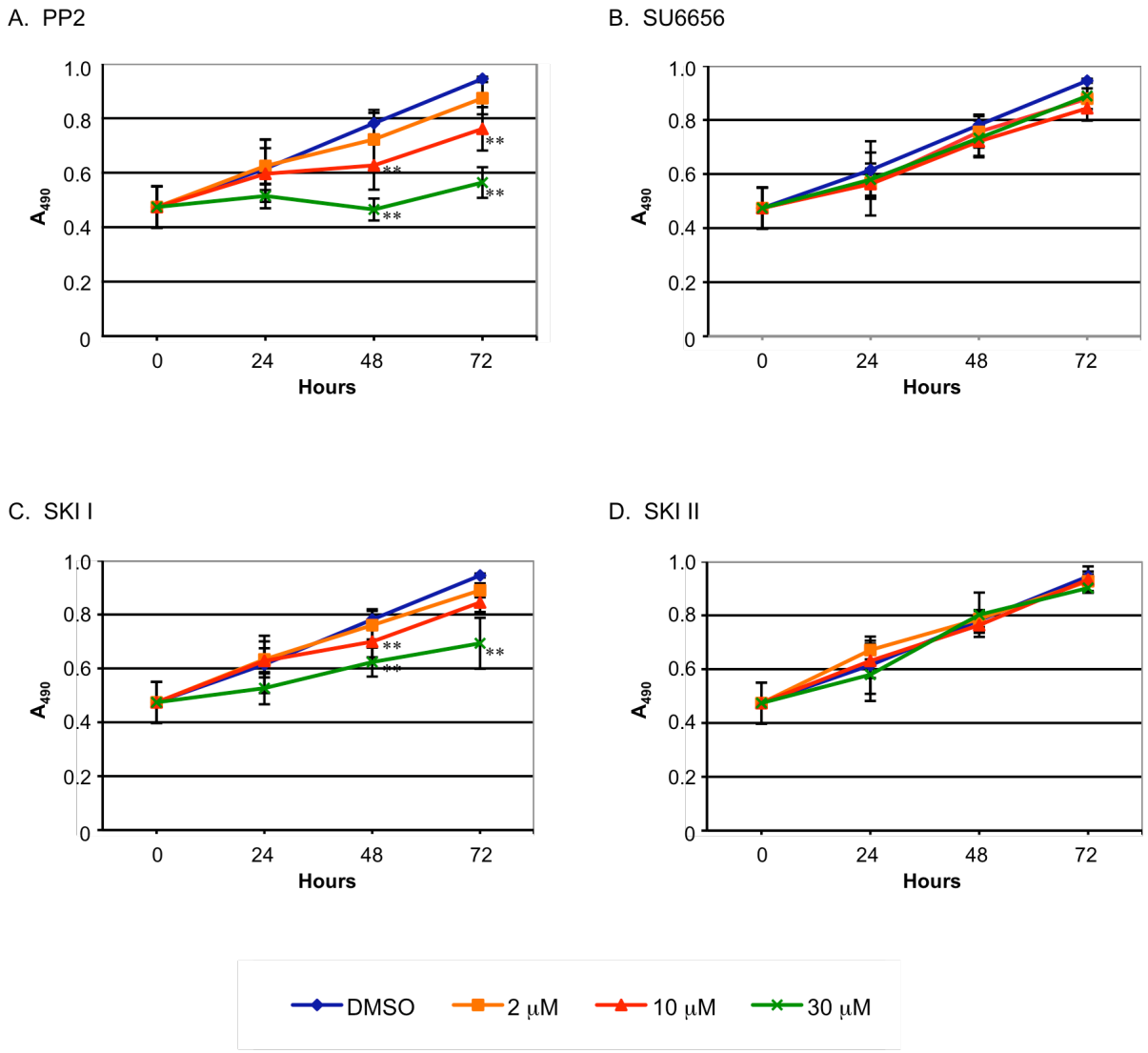


Figure 4.10. The proliferation of HT29 cells in the presence of Src family kinase inhibitors. HT29 cells were serum starved for 24 hours, following which 5000 cells were seeded per well of a 96 well plate. After 24 hours, cells were treated with the indicated concentrations of **A.** PP2, **B.** SU6656, **C.** SKI I, and **D.** SKI II, and MTS proliferation assays carried out at the time of treatment (0 hours), and at 24, 48, and 72 hours post-treatment. The graphs shown represent the average of three independent experiments done in triplicate, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the DMSO control is shown.

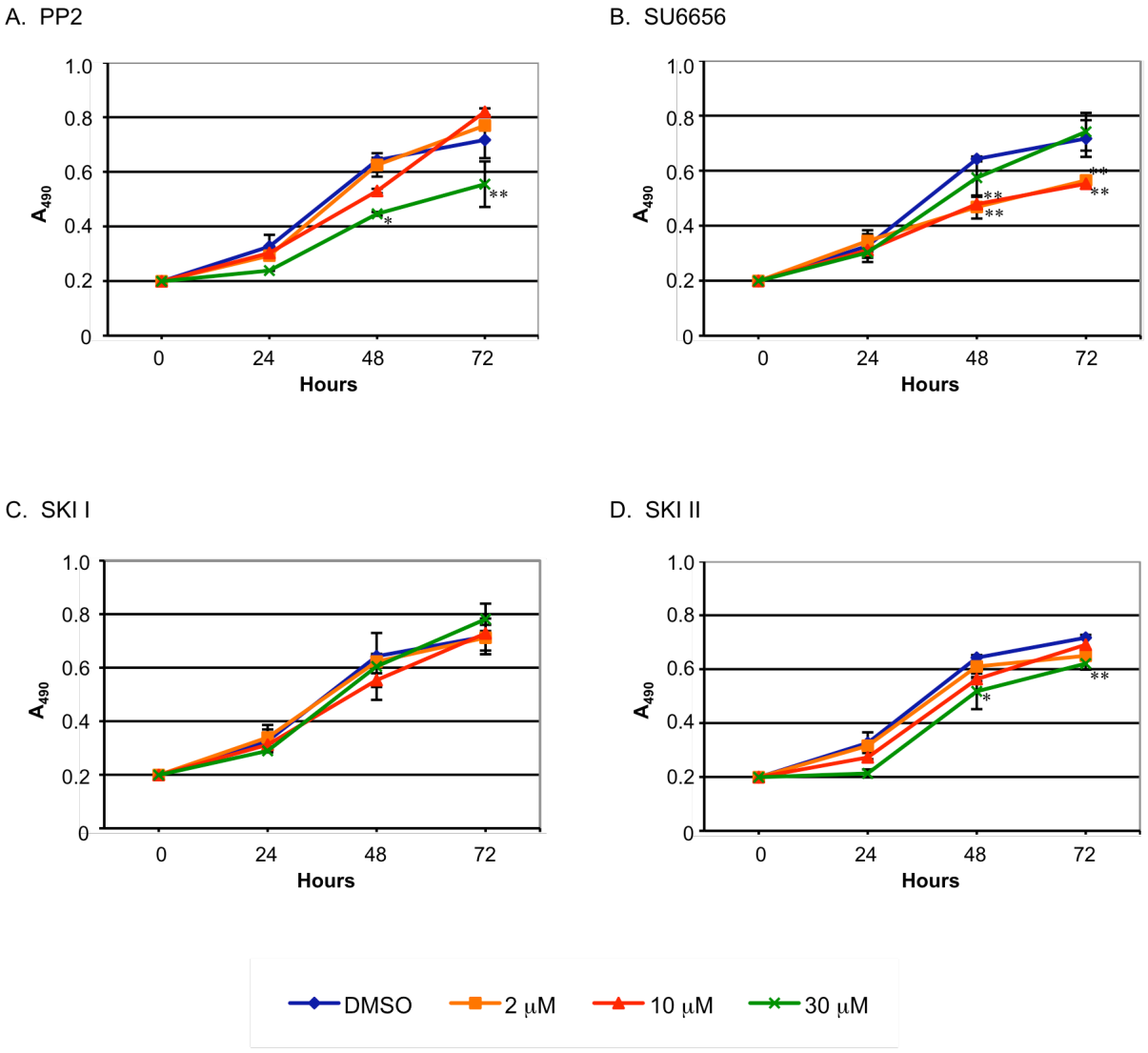


Figure 4.11. The proliferation of HCT116 cells in the presence of Src family kinase inhibitors. HCT116 cells were serum starved for 24 hours, following which 5 000 cells were seeded per well of a 96 well plate. After 24 hours, cells were treated with the indicated concentrations of **A.** PP2, **B.** SU6656, **C.** SKI I, and **D.** SKI II, and MTS proliferation assays carried out at the time of treatment (0 hours), and at 24, 48, and 72 hours post-treatment. The graphs shown represent the average of three independent experiments done in triplicate, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the DMSO control is shown.

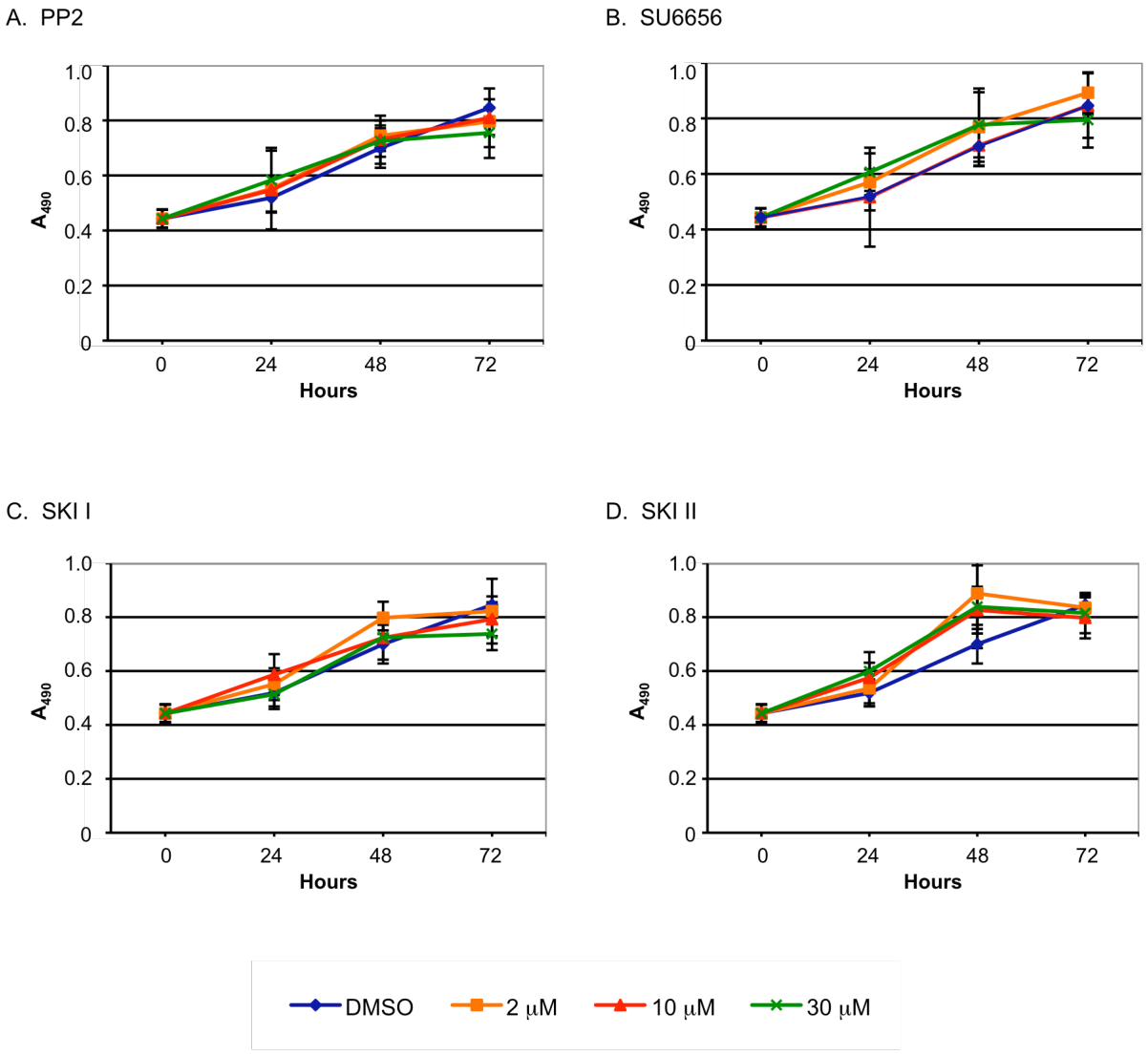


Figure 4.12. The proliferation of SW480 cells in the presence of Src family kinase inhibitors. SW480 cells were serum starved for 24 hours, following which 20 000 cells were seeded per well of a 96 well plate. After 24 hours, cells were treated with the indicated concentrations of **A.** PP2, **B.** SU6656, **C.** SKI I, and **D.** SKI II, and MTS proliferation assays carried out at the time of treatment (0 hours), and at 24, 48, and 72 hours post-treatment. The graphs shown represent the average of three independent experiments done in triplicate, with the standard deviation given as error.

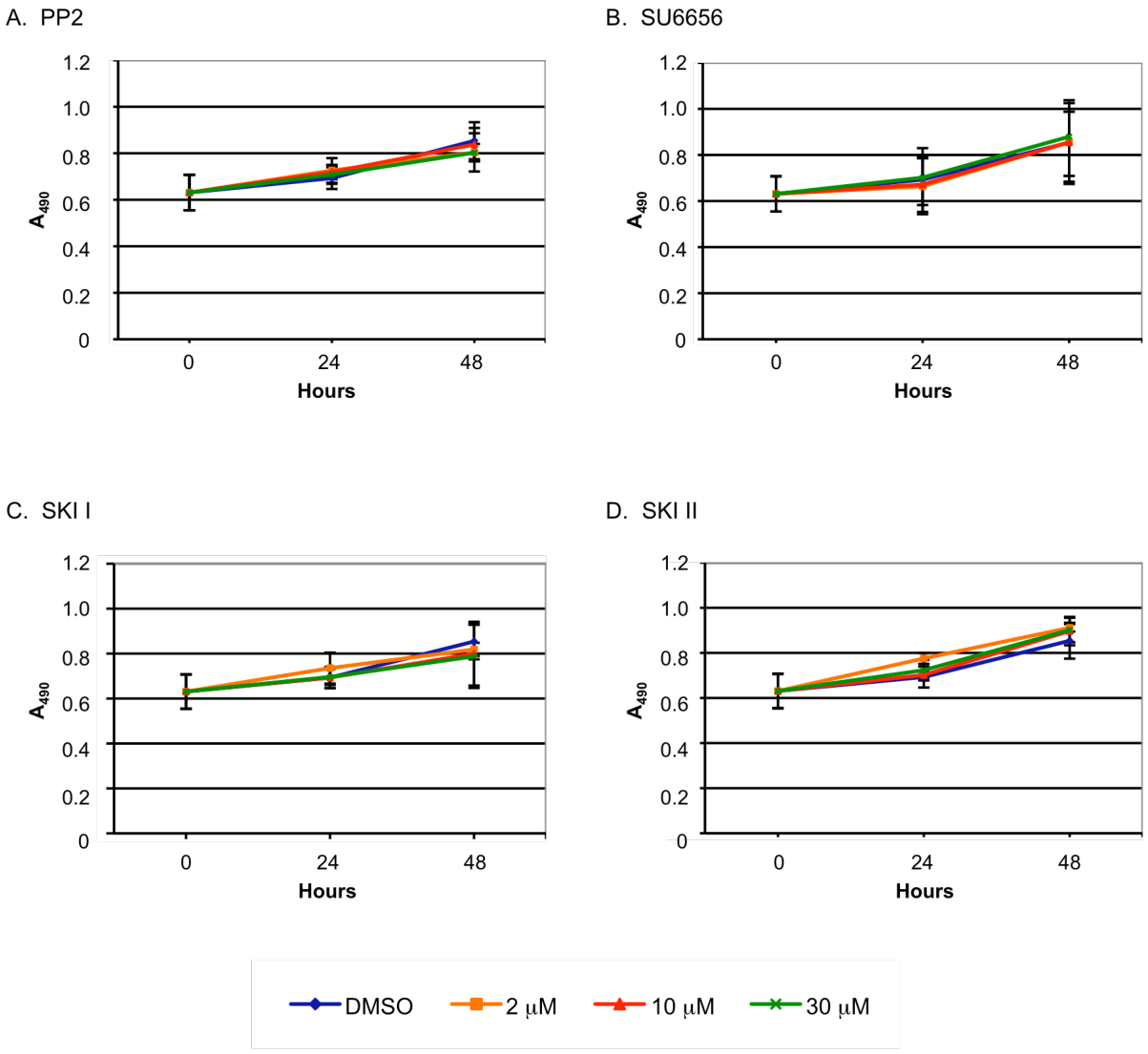


Figure 4.13. The proliferation of HepG2 cells in the presence of Src family kinase inhibitors. Cells were serum starved for 24 hours, following which 10 000 cells were seeded per well of a 96 well plate. After 24 hours, cells were treated with the indicated concentrations of **A.** PP2, **B.** SU6656, **C.** SKI I, and **D.** SKI II, and MTS proliferation assays carried out at the time of treatment (0 hours), and at 24 and 48 hours post-treatment. The graphs shown represent the average of three independent experiments done in triplicate, with the standard deviation given as error.

proliferation, whereby the number of cells present in each well was counted every 24 hours for 120 hours. A growth curve demonstrated that HT29 cells with decreased Src, Fyn, or Yes expression did indeed have reduced proliferation when compared to the control cells (Figure 4.14 B). Interestingly, both Src and Yes knockdown resulted in a decrease in cell number of approximately 50% by 120 hours, when compared to the control cells, while Fyn knockdown resulted in a decrease of 30%. This approach was then also used to examine the growth of HCT116 and SW480 cells.

When MTS assays were carried out on HCT116 cells, there appeared to be an increase in the proliferation of the parental HCT116 cells over the cells containing shRNA vectors. This difference in proliferation may, however, have been an artifact of being represented as fold growth, as fewer untransduced cells were present in the 0 hour timepoint in every assay. There was no statistically significant difference observed between the pGIPZ control cells and any of the shRNA expressing cells, however. Similar results were obtained when a cellular growth curve was made, as there was no significant difference in the growth of any of the cells (Figure 4.15 B).

Although SW480 cells with stable Src knockdown appeared to have a reproducible decrease in proliferation when compared to that of the parental SW480 cells and control pGIPZ cells, as measured with the MTS assay, this was not statistically significant (Figure 4.16 A). Similarly, no significant differences in proliferation were observed in any of the other knockdown cell lines. However, when a growth curve was created, SW480 cells with decreased Src expression were found to have decreased numbers of cells after 72 hours when compared to both the pGIPZ and parental control cells. The number of pGIPZ cells present at 120 hours was significantly fewer than the number of parental control cells, however. Therefore, although the growth of SW480 cells with decreased Fyn expression was significantly reduced when compared to the parental cells and cells with decreased Yes expression, between which there was no difference in proliferation, no significant difference in proliferation was observed between cells with decreased Fyn expression and the pGIPZ control cells (Figure 4.16 B).

In order to assess the proliferation of HepG2 cells with decreased SFK expression, they were transiently transfected with chemically synthesized siRNAs targeting the SFKs at the time of seeding, 24 hours prior to beginning the MTS assay. This was sufficient time to decrease Src protein levels nearly completely, although Yes protein levels are not significantly decreased

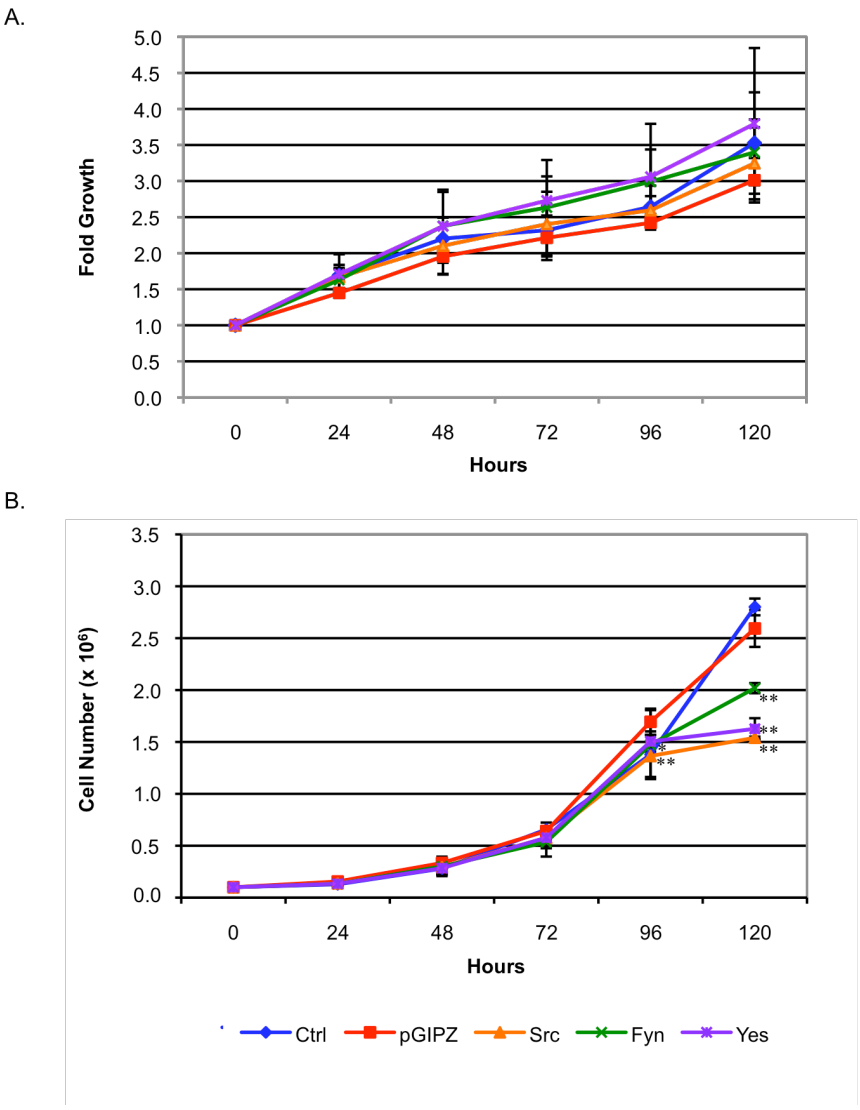


Figure 4.14. The proliferation of HT29 cells with decreased Src, Fyn, or Yes expression. **A.** Untransduced control HT29 cells (Ctrl), and HT29 cells transduced with an empty control vector (pGIPZ), or an shRNA vector targeting Src, Fyn, or Yes were serum starved for 24 hours, following which 5000 cells were seeded per well of a 96 well plate. MTS assays were carried out at the time of seeding (0 hour), and every 24 hours for five days. The graph shown represents the average fold growth of three independent experiments done in triplicate, with the standard deviation given as error. **B.** HT29 cells stably transduced with shRNA vectors, or untransduced HT29 control cells (Ctrl) were serum starved for 24 hours prior to 1.0 x 10^5 cells being seeded per 35 mm plate. Cells were trypsinized and counted every 24 hours using a Coulter Counter ZM for five days. The graph shown represents the average of two independent experiments done in duplicate, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the pGIPZ control is shown.

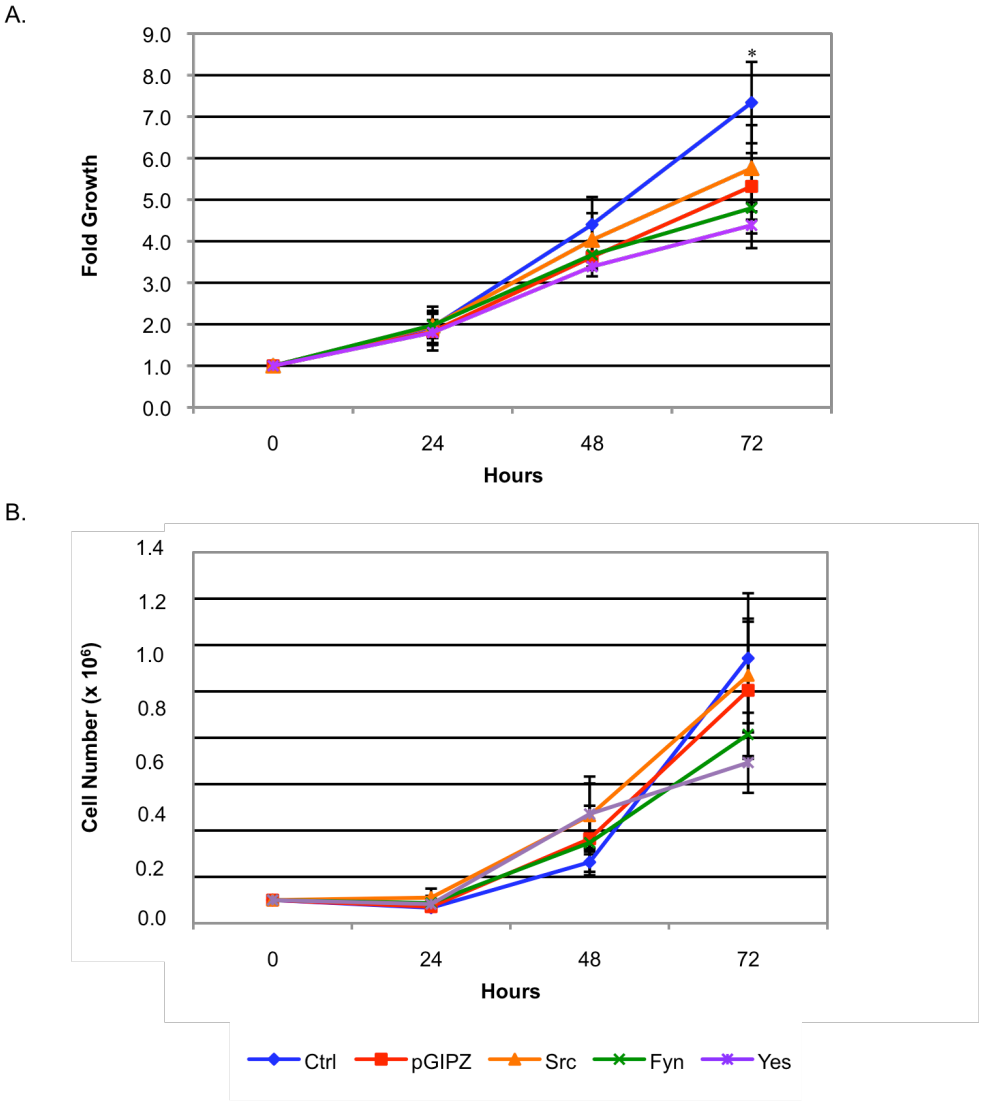


Figure 4.15. The proliferation of HCT116 cells with decreased Src, Fyn, or Yes expression. A. Untransduced control HCT116 cells (Ctrl), and HCT116 cells transduced with an empty control vector (pGIPZ), or an shRNA vector targeting Src, Fyn, or Yes were serum starved for 24 hours, following which 5000 cells were seeded per well of a 96 well plate. MTS assays were carried out at the time of seeding (0 hour), and at 24, 48, and 72 hours after seeding. The graph shown represents the average fold growth of four independent experiments done in triplicate, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) versus the pGIPZ control is shown. **B.** HCT116 cells stably transduced with shRNA vectors, or untransduced HCT116 control cells (Ctrl) were serum starved for 24 hours prior to 1.0 x 10^5 cells being seeded per 35 mm plate. Cells were trypsinized and counted using a Coulter Counter ZM every 24 hours up to 72 hours. The graph shown is the average of four independent experiments done in duplicate, with the standard deviation given as error.

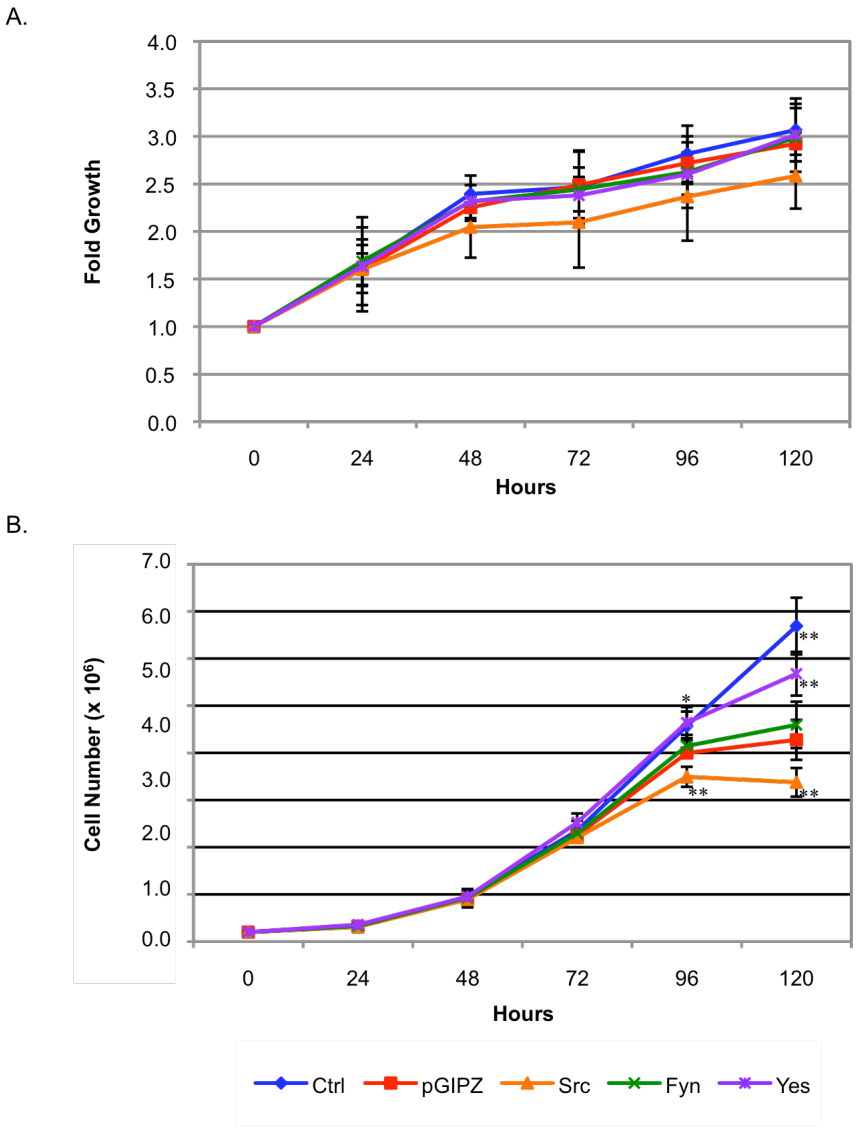


Figure 4.16. The proliferation of SW480 cells with decreased Src or Fyn expression. A. Untransduced control SW480 cells (Ctrl), and SW480 cells transduced with an empty control vector (pGIPZ), or an shRNA vector targeting Src, Fyn, or Yes were serum starved for 24 hours, following which 5000 cells were seeded per well of a 96 well plate. MTS assays were carried out at the time of seeding (0 hour), and every 24 hours for five days. The graph shown represents the average fold growth of three independent experiments done in triplicate, with the standard deviation given as error. **B.** SW480 cells stably transduced with shRNA vectors, or untransduced SW480 control cells (Ctrl) were serum starved for 24 hours prior to 1.0 x 10^5 cells being seeded per 35 mm plate. Cells were trypsinized and counted every 24 hours using a Coulter Counter ZM for five days. The graph shown represents the average of three independent experiments done in duplicate, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the pGIPZ control is shown.

until approximately 72 hours. No significant differences in proliferation were observed between the transfected and control cells, which corresponds with the finding that none of the SFK inhibitors tested had any effect on the proliferation of this cell line. A HepG2 growth curve was therefore not created. In addition to Src and Yes siRNA being used to reduce the expression of these proteins individually, HepG2 cells were also transfected with both siRNAs together in order to determine if their proliferation would be affected when Src and Yes levels were decreased simultaneously; however, no difference in proliferation was observed (Figure 4.17).

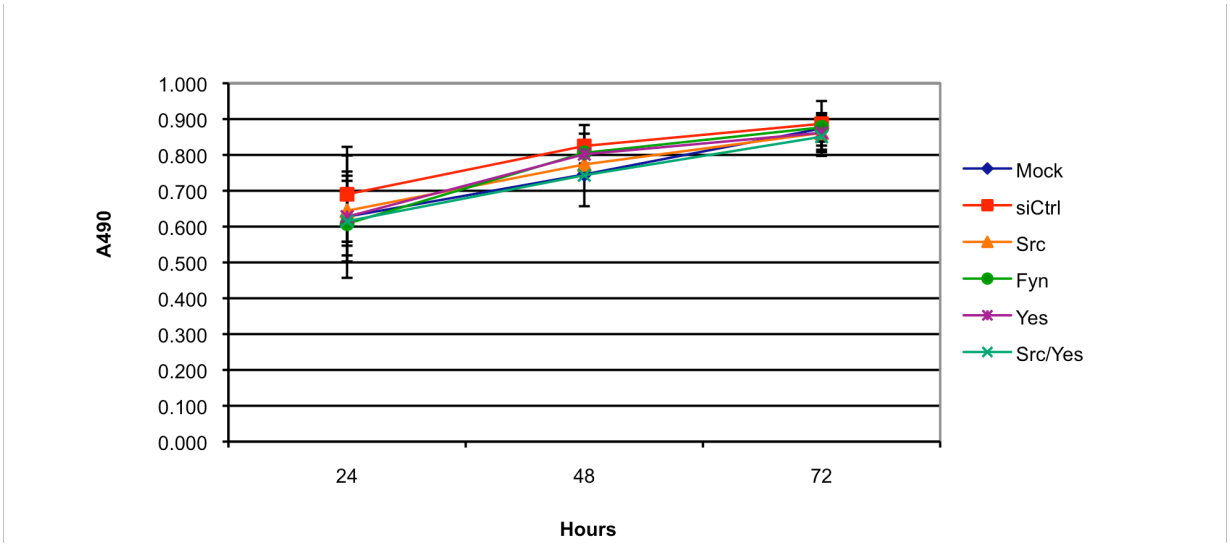


Figure 4.17. The proliferation of HepG2 cells with decreased Src, Fyn, or Yes expression. HepG2 cells were trypsinized, counted, and seeded at the time of reverse transfection with no siRNA (Mock), a scrambled control siRNA (siCtrl), or siRNAs targeting either Src, Yes, or Fyn individually, or Src and Yes together, in wells of a 96 well plate. MTS assays were carried out every 24 hours until 72 hours after seeding, at which time the cells had reached confluency. Data presented is the average of three independent experiments done in triplicate, with the standard deviation given as error.

4.3.1.3. Summary

Although multiple SFKs have been implicated in proliferation, results in the literature have been variable, and changes in SFK expression level or SFK inhibitor treatment have been found to affect the proliferation of some cell lines and not others. Our findings also suggest that the SFKs may affect proliferation in only a subset of cell lines, as the inhibitors used in these studies were found to significantly reduce the proliferation of only HT29 and HCT116 cells (Table 4.2). The ability of the SFK inhibitors to affect proliferation was also dependent upon the inhibitor being used. When the effects of SFK knockdown were examined, it was found that HT29 cells expressing Src, Yes and Fyn shRNA sequences had decreased growth when compared to control cells. In contrast, HCT116 cells, which had decreased proliferation when grown in the presence of the SFK inhibitors, showed no significant differences in growth when any one of the SFKs was knocked down. Furthermore, although none of the SFK inhibitors tested had an effect on the proliferation of SW480 cells, SW480 cells with decreased Src expression proliferated more slowly than the control cells. In HepG2 cells, however, neither decreases in Src or Yes expression nor Src kinase inhibition had an effect on proliferation. SFK involvement in the proliferation of cancer cell lines, therefore, appears to be cell line dependent.

Table 4.2. Summary of the effects of Src family kinase inhibitor treatment or decreased Src, Fyn, or Yes expression on the proliferation of selected cancer cell lines. Relative levels of inhibition of proliferation are given as follows: − No effect, +/− less than 20%, but statistically significant, inhibition, + 20-35% inhibition, ++ 35-50% inhibition.

	shRNA/siRNA			Inhibitors			
	Src	Fyn	Yes	PP2	SU6656	SKI I	SKI II
HT29	++	+	++	++	−	+	−
HCT116	−	−	−	++	+	−	+/−
SW480	++	−	−	−	−	+	−
HepG2	−	−	−	−	−	−	−

4.3.2. Cellular Adhesion to Fibronectin and Collagen

The adhesive properties of cancer cells to both other cells and the extracellular matrix (ECM) are generally altered, which contributes to their ability to spread. Although cell-cell adhesion was not investigated in this work, adhesion to both fibronectin and collagen, which are important components of the ECM, was investigated. In order to assess the adhesion of our cell lines of interest, cells were seeded into tissue culture wells that had previously been coated with either fibronectin or collagen. After allowing the cells to adhere to the wells for an hour, unadherent cells were washed from the wells, and the adherent cells fixed and stained so that the level of cellular adhesion could be assessed colourimetrically. Both cells that had been treated with SFK inhibitors and those with decreased Src, Fyn, or Yes expression were assessed for their ability to adhere to both fibronectin and collagen.

4.3.2.1. Effects of Src family kinase inhibitor treatment on cellular adhesion

In order to determine the effect of SFK inhibition on cellular adhesion to both fibronectin and collagen, HT29, SW480, HCT116, and HepG2 cells were treated with SFK inhibitors and examined for their ability to adhere to these ECM components. The four SFK inhibitors: PP2, SU6656, SKI I, and SKI II, were used at three different concentrations to inhibit the SFKs, as indicated. As three different concentrations were used from a single stock solution, this resulted in cells being exposed to different volumes of the DMSO solvent, depending on the concentration of inhibitor used. It was discovered in our adhesion assays that higher volumes of DMSO alone increased the adhesion of some of our cell lines of interest. To control for this effect, three different DMSO controls were used in each assay, whereby cells were treated with volumes of DMSO equivalent to those that were used to deliver each of the three concentrations of inhibitors. When analyzing data, it was therefore necessary to compare the adhesion of cells in the presence of a given concentration of inhibitor to the control cells treated with the corresponding volume equivalent of DMSO. Data is therefore presented as the percent migration of each sample in relation to its respective DMSO control.

In HT29 cells, it was found that treatment with the highest concentrations of PP2, SU6656, and SKI I decreased the adhesion of these cells to both fibronectin and collagen by at least 40%, while no decrease in adhesion was observed following SKI II treatment (Figure 4.18 A, B). Although treatment with all three concentrations of PP2 resulted in significantly decreased

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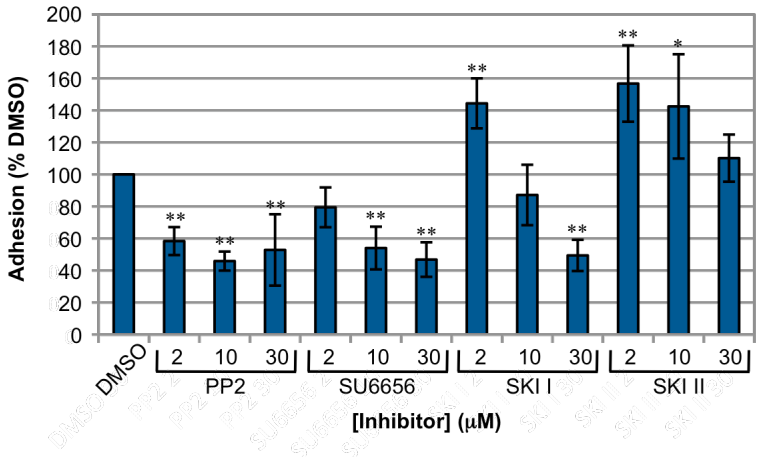

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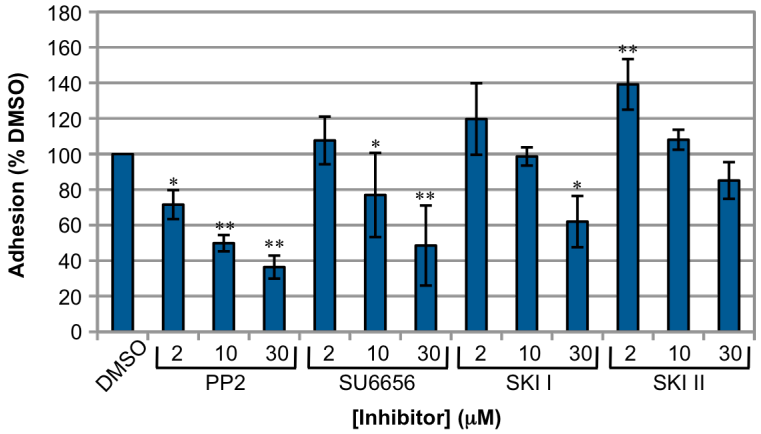


Figure 4.18. The adhesion of HT29 cells to fibronectin or collagen in the presence of Src family kinase inhibitors. HT29 cells were trypsinized, counted, and 1.0 x 10^5 cells seeded per well of a 96 well plate coated with either **A.** fibronectin or **B.** collagen in the presence of the indicated concentrations of the Src kinase inhibitors PP2, SU6656, SKI I or SKI II, or in the volume of DMSO equivalent to those concentrations. Following a one hour incubation, unadhered cells were washed from the plate, and the remaining cells fixed and stained with crystal violet for colourimetric analysis. The data presented are the mean of the percent level of adhesion, relative to the control cells treated with DMSO, of three independent experiments done in triplicate, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the DMSO control is shown.

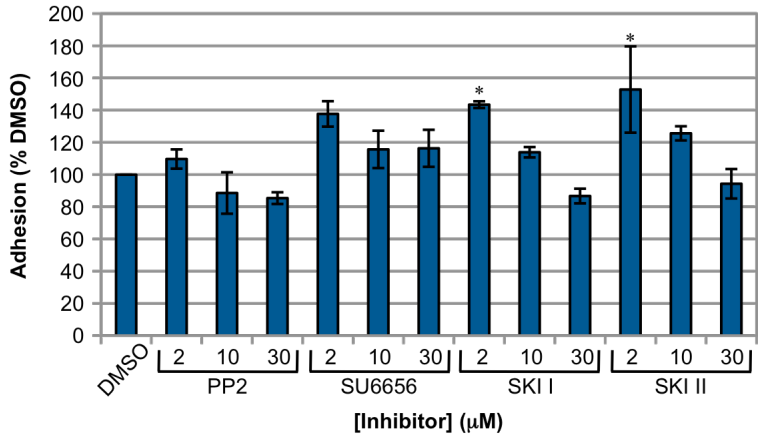
adhesion to both fibronectin and collagen, the effects of SU6656 and SKI I treatment were concentration dependent, with the greatest decrease observed using the highest concentration of inhibitor. Unexpectedly, the adhesion of HT29 cells to fibronectin was significantly increased by treatment with the lower concentrations of both SKI I and SKI II.

When the adhesion of HCT116 cells was examined, it was found that although treatment with 2 μM of SKI I or SKI II resulted in an increase in the adhesion of this cell line to fibronectin, no effects were observed when they were treated with either the higher concentrations of these inhibitors, or PP2 or SU6656 (Figure 4.19 A). In contrast, the adhesion of HCT116 cells to collagen was affected by treatment with three of the inhibitors investigated, whereby treatment with 30 μM of PP2, SKI I, and SKI II reduced the adhesion of these cells to collagen by 40%, 25%, and 25%, respectively. An increase in the adhesion of HCT116 cells to collagen in response to treatment with 2 μM of SKI I and SKI II was also observed. SU6656 treatment did not significantly affect HCT116 adhesion to collagen (Figure 4.19 B).

When the effect of SFK inhibition on the adhesion of SW480 cells was examined it was found that treatment with the highest concentration of PP2 tested resulted in a 50% decrease in adhesion to fibronectin, while none of the other inhibitors tested had any effect (Figure 4.20 A). Rather, as was seen in HT29 cells, treatment with 2 μM of SKI I or SKI II resulted in an increase in adhesion to fibronectin, although this effect was not observed when higher concentrations of these inhibitors were used. When the adhesion of SW480 cells to collagen was examined, 30 μM of PP2 was again found to reduce the adhesion of these cells by 35% (Figure 4.20 B). This was also observed in cells treated with 30 μM of SKI I, whereby their adhesion to collagen was decreased by 30%. The adhesion of SW480 cells to collagen was increased by treatment with all three concentrations of SU6656, while only the lowest concentration of SKI II significantly increased the adhesion of these cells.

Finally, the treatment of HepG2 cells with PP2, SU6656, and SKI I resulted in significant, concentration dependent decreases in the adhesion of these cells to fibronectin, with 30 μM of these inhibitors reducing adhesion by 40%, 35%, and 70%, respectively. In contrast, SKI II was not observed to have any statistically significant effect on the adhesion of HepG2 cells to fibronectin (Figure 4.21 A). PP2 and SKI I were also able to significantly reduce HepG2 adhesion to collagen by 20%, although only at the highest concentration used (Figure 4.21 B). An increase in the adhesion of HepG2 cells to collagen was also observed in response to

A.



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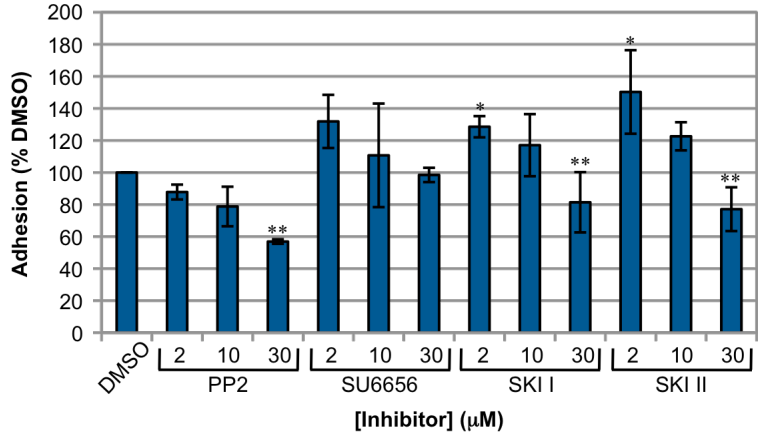
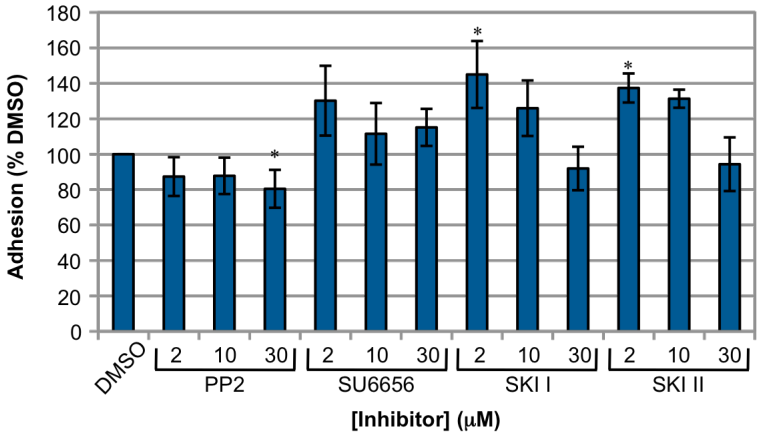


Figure 4.19. The adhesion of HCT116 cells to fibronectin or collagen in the presence of Src family kinase inhibitors. HCT116 cells were trypsinized, counted, and 5.0 x 10^4 cells seeded per well of a 96 well plate coated with either **A.** fibronectin or **B.** collagen in the presence of the indicated concentrations of the Src kinase inhibitors PP2, SU6656, SKI I or SKI II, or in the volume of DMSO equivalent to those concentrations. Following a one hour incubation, unadhered cells were washed from the plate, and the remaining cells fixed and stained with crystal violet for colourimetric analysis. The data presented are the mean of the percent level of adhesion, relative to the control cells treated with DMSO, of three independent experiments done in triplicate, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the DMSO control is shown.

A.



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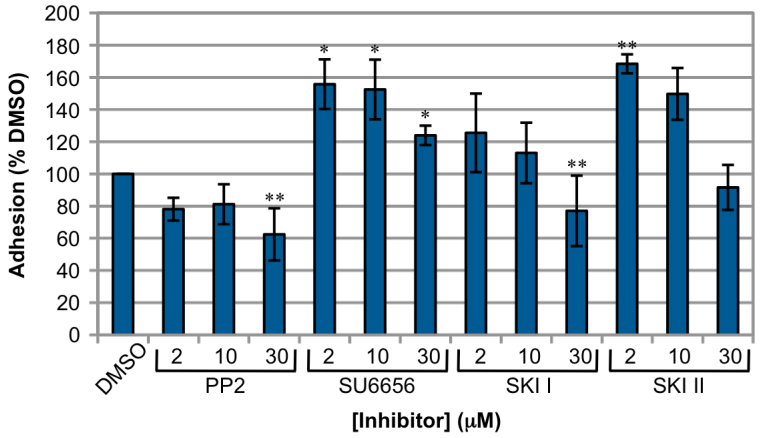

Figure 4.20. The adhesion of SW480 cells to fibronectin or collagen in the presence of Src family kinase inhibitors. SW480 cells were trypsinized, counted, and 1.5 x 10^5 cells seeded per well of a 96 well plate coated with either **A.** fibronectin or **B.** collagen in the presence of the indicated concentrations of the Src kinase inhibitors PP2, SU6656, SKI I or SKI II, or in the volume of DMSO equivalent to those concentrations. Following a one hour incubation, unadhered cells were washed from the plate, and the remaining cells fixed and stained with crystal violet for colourimetric analysis. The data presented are the mean of the percent level of adhesion, relative to the control cells treated with DMSO, of three independent experiments done in triplicate, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the DMSO control is shown.

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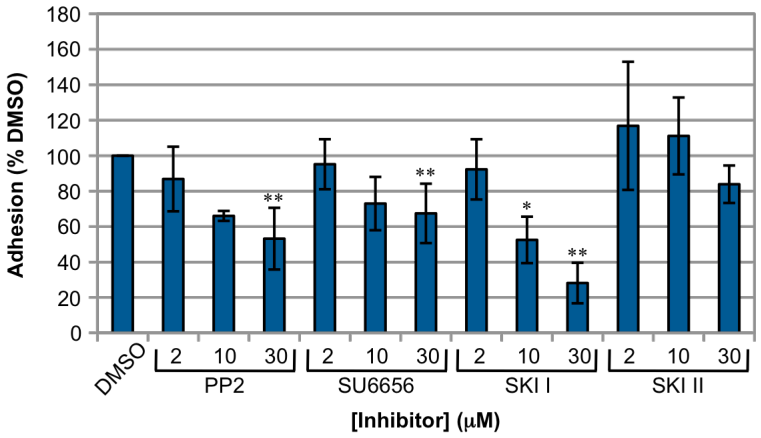

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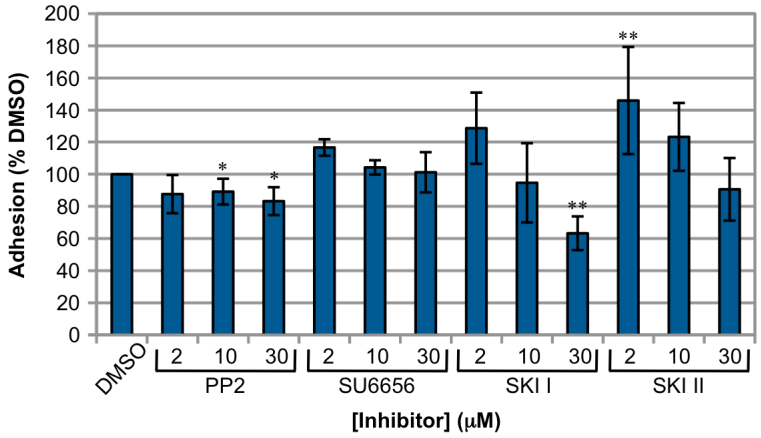


Figure 4.21. The adhesion of HepG2 cells to fibronectin or collagen in the presence of Src family kinase inhibitors. HepG2 cells were trypsinized, counted, and 5.0 x 10^4 cells seeded per well of a 96 well plate coated with either **A.** fibronectin or **B.** collagen in the presence of the indicated concentrations of the Src kinase inhibitors PP2, SU6656, SKI I or SKI II, or in the volume of DMSO equivalent to those concentrations. Following a one hour incubation, unadhered cells were washed from the plate, and the remaining cells fixed and stained with crystal violet for colourimetric analysis. The data presented are the mean of the percent level of adhesion, relative to the control cells treated with DMSO, of three independent experiments done in triplicate, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the DMSO control is shown.

treatment with 2 μM of SKI II. Therefore, the effects of the SFK inhibitors on the adhesion of the different cancer cell lines depended both upon both the inhibitor and cell line being investigated.

4.3.2.2. Effects of decreased Src, Fyn, or Yes expression on cellular adhesion

As the adhesion of all of our cell lines of interest to both fibronectin and collagen was decreased in response to treatment with at least one of the SFK inhibitors, we next examined the effects of Src, Fyn, and Yes knockdown on adhesion in order to determine if the inhibition of multiple SFKs results in greater effects on adhesion than decreases in the expression of a single SFK. Although the level of adhesion of HT29 cells with decreased Src or Yes expression to fibronectin was reduced by 35% when compared to that of cells containing the pGIPZ control vector, no effect was observed in HT29 cells with decreased Fyn expression (Figure 4.22 A). Furthermore, although SFK inhibitor treatment resulted in a significant reduction in the adhesion of HT29 cells to collagen, no such effect was observed as a result of decreased Src, Fyn, or Yes expression in these cells (Figure 4.22 A). Likewise, neither the adhesion of HCT116 (Figure 4.22 B), nor SW480 cells (Figure 4.22 C) with decreased Src, Fyn, or Yes expression to either fibronectin or collagen was significantly reduced when compared to that of the cells transduced with the pGIPZ control vector.

HepG2 cells transiently transfected with a control scrambled siRNA, or siRNAs targeting Src, Yes, or Fyn were also assayed for their ability to adhere to fibronectin and collagen. The adhesion of HepG2 cells with decreased Src and Yes expression to fibronectin was reduced by 30% and 25%, respectively, when compared to that of the control cells transfected with the scrambled siRNA (Figure 4.23 A). Similarly, the adhesion of HepG2 cells to collagen was reduced by 40% in response to decreased Yes expression, although decreases in Src expression did not have a statistically significant effect on the adhesion of these cells to collagen (Figure 4.23 B). When the adhesion of HepG2 cells expressing decreased levels of both Src and Yes in conjunction was investigated, it was found that there was no significant effect on the adhesion of these cells to either fibronectin or collagen (Figure 4.23 A, B).

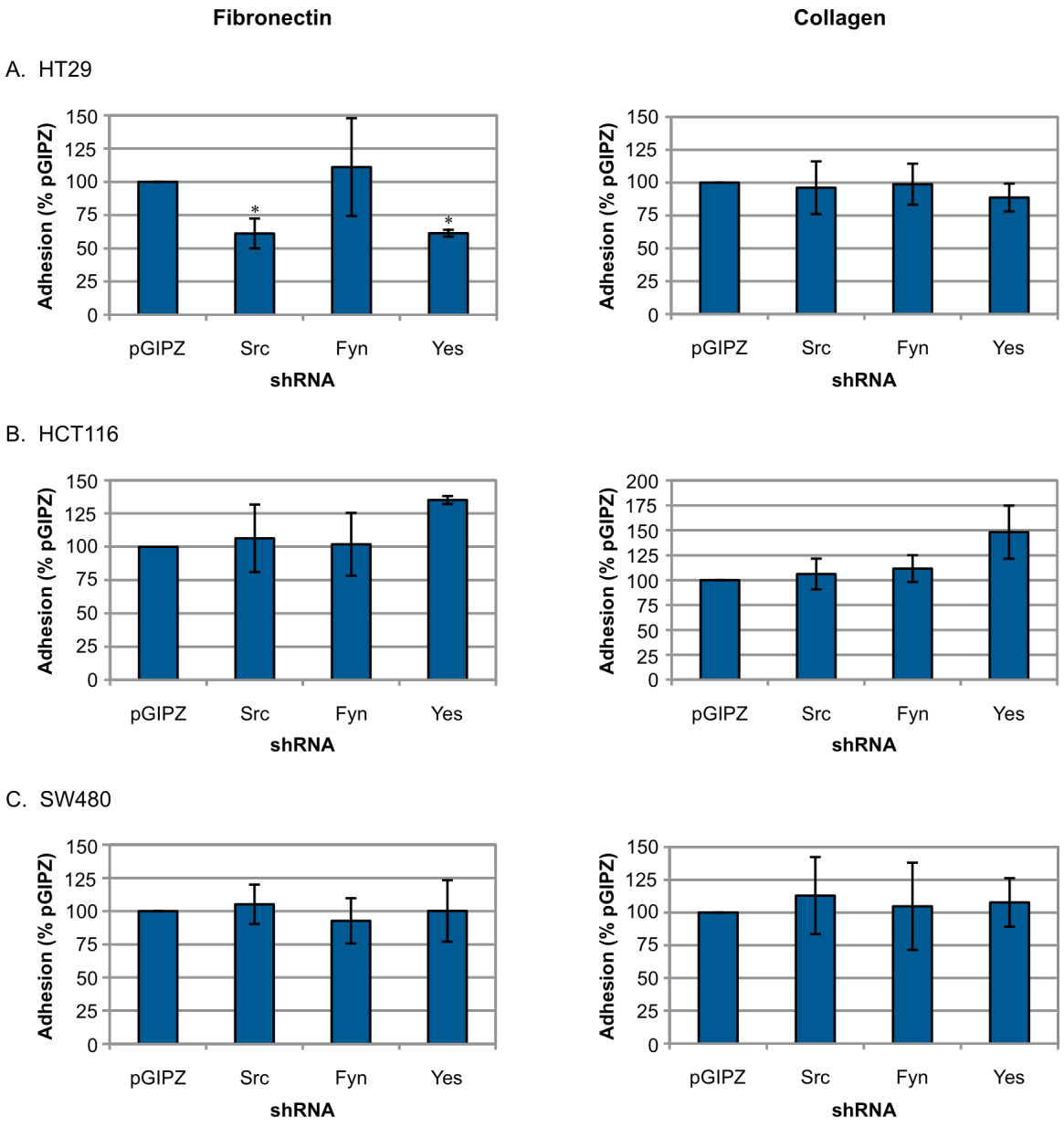


Figure 4.22. The adhesion of HT29, HCT116, and SW480 cells with decreased Src, Fyn, or Yes expression to fibronectin or collagen. Stably transduced **A.** HT29, **B.** HCT116, or **C.** SW480 cells were trypsinized, counted, and 1.0 x 10^5 HT29 cells, 5.0 x 10^4 HCT116 cells, or 1.5 x 10^5 SW480 cells seeded per well of a 96 well plate coated with either fibronectin or collagen. Following a one hour incubation, unadhered cells were washed from the plate, and the remaining cells fixed and stained with crystal violet for colourimetric analysis. The data presented are the mean of the percent level of adhesion, relative to that of the pGIPZ control cells, of at least three independent experiments done in triplicate. The standard deviation is given as error. Statistical significance with a *P* value of less than 0.05 (*) versus the pGIPZ control is shown.

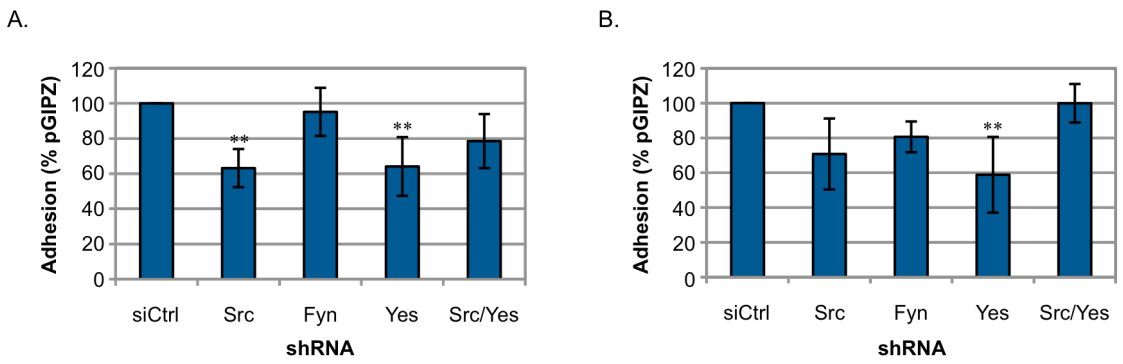


Figure 4.23. The adhesion of HepG2 cells with decreased Src, Fyn, or Yes expression to fibronectin or collagen. HepG2 cells were transfected with either a scrambled control siRNA (siCtrl), or siRNAs targeting Src, Fyn, or Yes individually, or Src and Yes in conjunction. Cells were grown for five days before 5.0 x 10^4 cells were seeded in a 96 well plate coated with either **A.** fibronectin or **B.** collagen. Following a one hour incubation, unadhered cells were washed from the plate, and the remaining cells fixed and stained with crystal violet for colourimetric analysis. The data presented are the mean of the percent level of adhesion, relative to that of the scrambled control cells, of four (fibronectin) or three (collagen) independent experiments done in triplicate, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the control is shown.

4.3.2.3. Summary

The effect of SFK inhibition on adhesion appears to be dependent upon both the inhibitor and cell line being investigated, as well as upon the adhesion molecule being assayed (Table 4.3). In particular, HT29 cells had the most significantly decreased adhesion to both fibronectin and collagen when treated with SFK inhibitors, as well as when Src and Yes protein levels were decreased. While the adhesion of the majority of the cell lines to both fibronectin and collagen was inhibited by treatment with either PP2 or SKI I, SU6656 and SKI II only inhibited the adhesion of HCT116 cells. Furthermore, treatment with a number of the inhibitors resulted in the increased adhesion of given cell lines to either fibronectin or collagen. In particular, this was observed when cells were treated with the lowest concentration of the inhibitors investigated in this study, and although this was observed mostly in response to SKI I and SKI II treatment, SU6656 also had this effect on SW480 cells. Although the adhesion of all four of the cell lines investigated to either fibronectin or collagen was inhibited by at least one SFK inhibitor, decreases in the expression of individual SFKs did not have as significant

an effect. Only HT29 and HepG2 cells with decreased Src and Yes expression had reduced adhesion to fibronectin, while only HepG2 cells with decreased Yes expression had significantly reduced adhesion to collagen. Decreases in the expression of both Src and Yes in conjunction did not, however, affect the adhesion of HepG2 cells to either fibronectin or collagen.

Table 4.3. Summary of the effects of Src family kinase inhibitor treatment or decreased Src, Fyn or Yes expression on the adhesion of selected cancer cell lines to fibronectin and collagen. Relative levels of inhibition of adhesion to A. Fibronectin and B. Collagen in the presence of 30 µM of the indicated inhibitors are given as follows: − No effect, + 20-35% inhibition, ++ 35-50% inhibition, +++ more than 50% inhibition. Increased adhesion due to treatment with 2 µM of the inhibitors is indicated by an arrow.

A.

	shRNA/siRNA			Inhibitors			
	Src	Fyn	Yes	PP2	SU6656	SKI I	SKI II
HT29	++	−	++	+++	+++	↑/+++	↑/−
HCT116	−	−	−	−	−	↑/−	↑/−
SW480	−	−	−	+	−	↑/−	↑/−
HepG2	+	−	+	++	+	+++	−

B.

	shRNA/siRNA			Inhibitors			
	Src	Fyn	Yes	PP2	SU6656	SKI I	SKI II
HT29	−	−	−	+++	+++	++	↑/−
HCT116	−	−	−	++	−	↑/+	↑/+
SW480	−	−	−	++	↑/−	+	↑/−
HepG2	−	−	+	+	−	+	↑/−

4.3.3. Cellular Migration

Many of the SFKs have been strongly implicated in cellular migration. It would therefore be expected that cellular migration would be inhibited following either treatment with an SFK inhibitor, or in response to decreased SFK expression. The migratory capacity of cells that

were either treated with SFK inhibitors or had decreased Src, Fyn, or Yes expression was therefore assessed by performing wound healing assays. In these assays, cells were grown on etched glass cover slips until they were nearly confluent, at which time a wound was created in the monolayer. The wound was photographed at distinct locations on the cover slip both at the time of wounding, and again after the cells had been allowed to grow into the wound; the extent to which the cells had migrated into the wound was determined by measuring the width of the wound at both the time of wounding and following incubation. Representative images of SFK inhibitor treated HT29 cells showing wounds at 0 hours and 48 hours are shown in Figure 4.24. Due to the time consuming nature of the quantification, only one concentration of each inhibitor was used in the wound healing assays: 30 μM PP2, 2 μM SU6656, 10 μM SKI I, and 20 μM SKI II, as these concentrations are typically used in the literature (Jones *et al*., 2002; Golubovskaya *et al*., 2003; Boyd *et al*., 2004; Chiang *et al*., 2005; Matsumoto *et al*., 2010). Control cells treated with DMSO were also included in every assay, and the data presented as the percent of the wound that had closed following incubation, in relation to these control cells. HCT116 migration was not investigated using this assay because it was not possible to make a uniform, thin wound in a confluent monolayer of these cells.

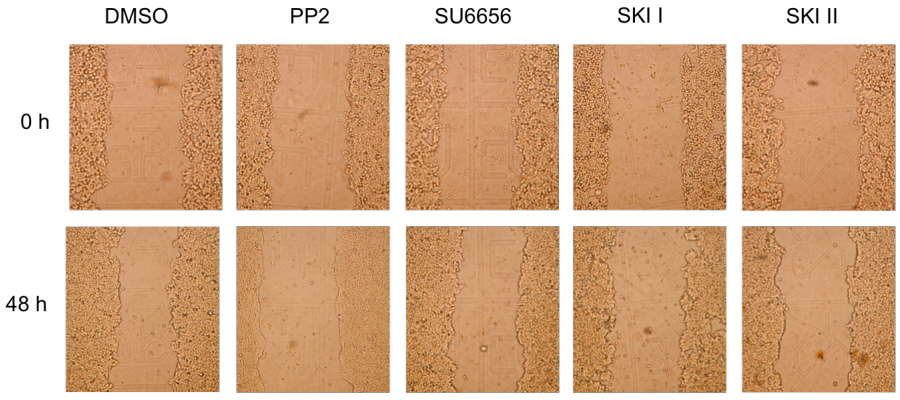


Figure 4.24. Representative images of an HT29 wound healing assay carried out in the presence of Src family kinase inhibitors. HT29 cells were seeded on etched glass cover slips in six well plates and grown to confluency. Upon reaching confluency, the cells were serum starved for 24 hours prior to being wounded using a 10 μL pipette tip. Photos were taken of the wound widths at the time of wounding (0 h) and the cells grown in media containing serum in the presence of DMSO or the Src kinase inhibitors at the following concentrations: 30 μM PP2, 2 μM SU6656, 10 μM SKI I, and 20 μM SKI II for 48 hours. Following 48 hours, photos were taken at the same locations as the 0 h photos, as indicated by the etched grid position (48 h).

4.3.3.1. Effects of Src family kinase inhibitor treatment on migration

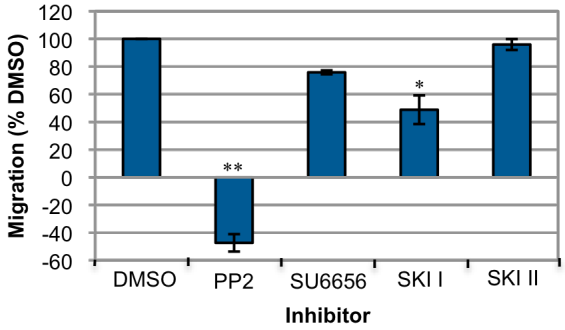
When the effect of SFK inhibition on migration was investigated, it was found that the four different SFK inhibitors affected cellular migration in a cell line and inhibitor specific manner. In the case of HT29 cells, PP2 completely prevented the migration of the cells into the wound; instead, it caused a change in the morphology of the cells that resulted in a wider wound at the later time point than was initially made in the monolayer. SU6656 treatment did not result in a statistically significant decrease in HT29 migration when compared to the control cells treated with DMSO. Treatment with SKI I was found to cause a 50% decrease in HT29 migration when compared to the control cells, while treatment with SKI II did not cause any significant differences in migration (Figure 4.25 A). Similar to what was observed with HT29 cells, the migration of SW480 cells was found to be completely inhibited by PP2 treatment, while SKI I treatment inhibited the migration of these cells into wounds by 60%. Treatment with either SU6656 or SKI II did not statistically inhibit SW480 migration (Figure 4.25 B).

The different SFK inhibitors also inhibited the migration of HepG2 cells. PP2 was once again found to reduce migration to the greatest extent; however, PP2 inhibited HepG2 migration by only 70%, compared with the complete inhibition of the other cell lines examined. SU6656 and SKI I treatment both inhibited HepG2 migration by 30%, while SKI II treatment did not result in a significant change in the migration of these cells in relation to the control cells treated with DMSO (Figure 4.25 C). Although the extent to which the SFK inhibitors reduced migration depended upon cell line, the migration of all of the cell lines investigated was inhibited by both PP2 and SKI I treatment.

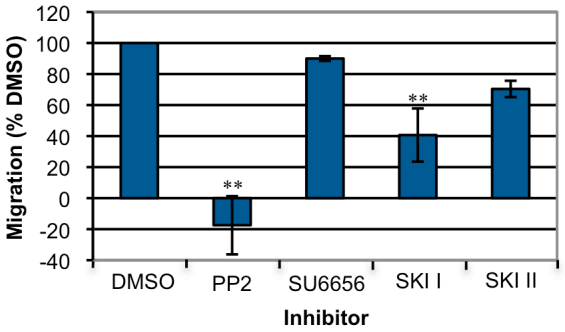
4.3.3.2. Effects of decreased Src, Fyn, or Yes expression on migration

After investigating the effects of SFK inhibition on migration, we next examined the effects of decreasing the expression of individual SFKs. The migration of HT29 cells with decreased Src, Fyn, or Yes expression was reduced by 30% to 45% when compared to the pGIPZ control cells (Figure 4.26 A). In SW480 cells, it was found that while decreased Src expression resulted in a reduction in migration of approximately 40%, neither decreased Fyn nor Yes expression had any significant effect on the migration of these cells (Figure 4.26 B). Finally, the migration of HepG2 cells was not affected by decreased Src, Fyn, or Yes expression, or decreased levels of both Src and Yes together (Figure 4.26 C).

A. HT29



B. SW480



C. HepG2

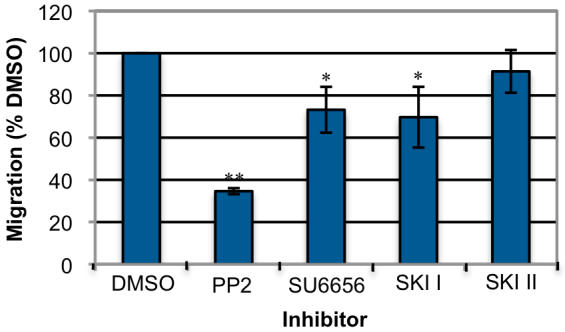


Figure 4.25. The migration of HT29, SW480, and HepG2 cells in the presence of Src family kinase inhibitors. A. HT29, **B.** SW480, or **C.** HepG2 cells were trypsinized, counted, and seeded into six well plates containing etched glass slides. Cells were grown to near confluency before being serum starved for 24 hours prior to three wounds being made in each monolayer. Each wound was photographed at two distinct locations at the time of wounding, and at the same locations after the cells had been grown under normal cell culture conditions for 24 to 48 hours. The width of the wound was measured at both timepoints to determine migration. The data is presented as the percent of the wound width that had closed during incubation, in relation to that of the DMSO control cells. Error bars are the standard deviation of at least two (HT29 and SW480) or three (HepG2) independent experiments. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the DMSO control is shown.

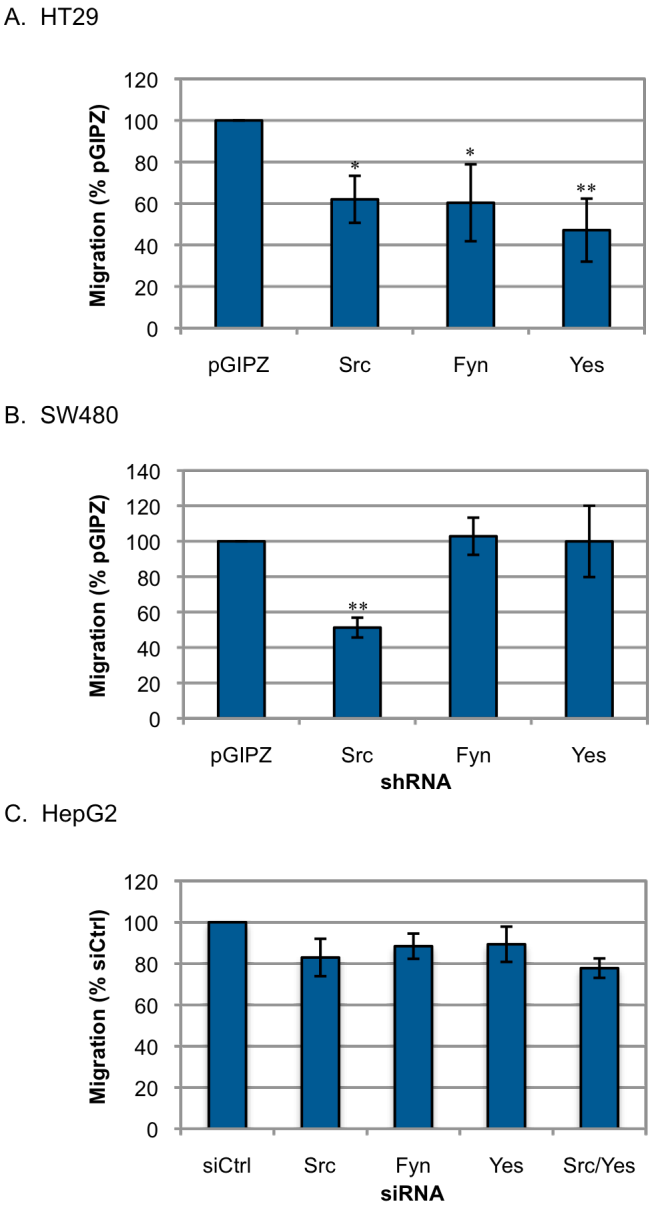


Figure 4.26. The migration of HT29, SW480, and HepG2 cells with decreased Src, Fyn, or Yes expression. A. HT29 or **B.** SW480 cells stably transduced with shRNA vectors targeting Src, Fyn, Yes, or the control vector pGIPZ were trypsinized, counted, and seeded into six well plates containing etched glass slides. **C.** HepG2 cells were seeded and transfected concurrently with chemically synthesized siRNAs targeting Src, Fyn, Yes, or a scrambled control siRNA (siCtrl). Cells were grown to near confluency before being serum starved for 24 hours prior to three wounds being made in each monolayer. Each wound was photographed at two distinct locations at the time of wounding, and at the same locations after the cells had been grown under normal cell culture conditions for 24 to 48 hours. The width of the wound was measured at both timepoints to determine migration. The data is presented as the percent of the wound width that had closed during incubation, in relation to that of the pGIPZ or siCtrl control cells, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the pGIPZ or siCtrl controls is shown.

4.3.3.3. Summary

Of the SFK inhibitors tested, PP2 elicited the greatest inhibition of migration in all of the cell lines investigated, as it completely prevented any growth into the wound area in both HT29 and SW480 cells, and decreased the migration of HepG2 cells by 70%. SKI I treatment also resulted in decreases in cellular migration in all three of the cell lines tested, while SU6656 inhibited the migration of HepG2 cells. SKI II did not inhibit the migration of any of the cell lines investigated. In addition, the migration of HepG2 cells was, in general, not affected by SFK inhibitor treatment to the same extent as HT29 and SW480 cells (Table 4.4). Therefore, while the extent to which the SFK inhibitor treatment reduced migration depended upon cell line, SFK inhibition resulted in reduced migration of all of the cell lines investigated.

When compared to the decreases in migration observed in the cell lines treated with SFK inhibitors, the effects on migration observed when the expression of a single SFK was decreased were small. While decreases in the expression of Src, Fyn, and Yes inhibited the migration of HT29 cells, when compared to the pGIPZ control cells, only decreased Src expression inhibited the migration of SW480 cells. Interestingly, despite the effects of SFK inhibition in HepG2 cells, no significant changes were observed in the migration of HepG2 cells with decreased Src, Fyn, or Yes expression (Table 4.4), suggesting that multiple SFKs may need to be targeted in order to inhibit migration in some cell lines.

Table 4.4. Summary of the effects of Src kinase inhibitor treatment or decreased Src, Fyn, or Yes expression on the migration of selected cancer cell lines. Relative levels of inhibition of proliferation are given as follows: − No effect, + 25-50% inhibition, ++ 50-75% inhibition, +++ greater than 75% inhibition.

	shRNA/siRNA			Inhibitors			
	Src	Fyn	Yes	PP2	SU6656	SKI I	SKI II
HT29	+	+	+	+++	−	++	−
SW480	++	−	−	+++	−	++	−
HepG2	−	−	−	++	+	+	−

4.3.4. Colony Forming Ability

Only cells that are able to grow in the absence of adhesion, as well as with a lack of contact inhibition are able to form colonies in soft agar from a single cell suspension. As these characteristics are an indication of transformation, soft agar colony forming assays can be used to assess the number of transformed cells within a population. In order to investigate the role of the SFKs in the ability of HT29 and HepG2 cells to form colonies, cells that were either treated with SFK inhibitors or expressed decreased levels of Src, Fyn, or Yes were seeded into soft agar and allowed to form colonies over three weeks for HT29 cells and two weeks for HepG2 cells. The number of colonies formed was then counted using a grid under a light microscope. Only HT29 and HepG2 cells were examined for the ability to form colonies in soft agar, as only these cell lines were found to form significant numbers of colonies.

4.3.4.1. Effects of Src family kinase inhibitor treatment on colony forming ability

The ability of HT29 and HepG2 cells to form colonies in soft agar in the presence of SFK inhibitors was investigated in order to examine the involvement of the SFKs in this process. As increasing amounts of DMSO resulted in an increase in the number of HT29 colonies formed, the different inhibitor concentrations were compared to the DMSO controls corresponding to the volume of inhibitor used, and the data presented as the percent of colonies formed when compared to the DMSO treated cells. It was found that the presence of 30 μM PP2 completely inhibited the formation of HT29 colonies, while 2 μM and 10 μM did not result in a decrease in colony number; rather, there was a statistically significant increase in colony number in the presence of low concentrations of PP2. Treatment with all concentrations of SU6656 significantly inhibited colony formation, however, with essentially no colonies able to grow in the presence of 10 μM or 30 μM of this inhibitor. SKI I was also able to significantly inhibit colony formation by HT29 cells, while the presence of SKI II had no statistically significant effect (Figure 4.27 A).

Unlike HT29 cells, the colony forming ability of HepG2 cells was not affected by the amount of DMSO present, as all of the control wells contained equal numbers of colonies. In this cell line, PP2 was able to inhibit the formation of colonies in a concentration dependent manner, with 2 μM having no effect and 30 μM causing the greatest inhibition. Likewise, although treatment with 2 μM of SU6656 was sufficient to produce a significant decrease in

colony formation, higher concentrations of this inhibitor decreased colony formation to a greater extent. As in HT29 cells, SU6656 had a greater effect on HepG2 colony formation than PP2. No effect on the colony forming ability of HepG2 cells was observed in the presence of either SKI I or SKI II (Figure 4.27 B). Therefore, as with the other cellular phenotypes investigated, the ability of the SFK inhibitors to reduce colony formation depended upon the SFK inhibitor being used, as well as the cell line being treated.

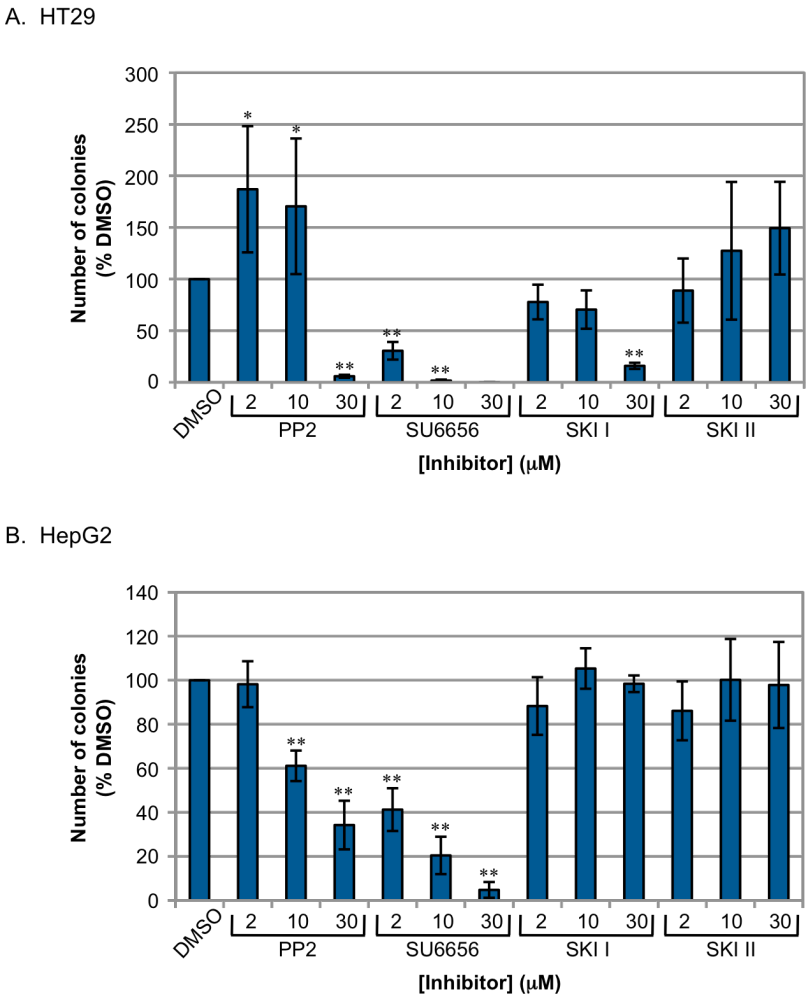


Figure 4.27. The colony forming ability of HT29 and HepG2 cells in the presence of Src family kinase inhibitors. A. HT29 and **B.** HepG2 cells were trypsinized, counted, and 5000 cells seeded in 0.3% soft agarose in the presence of the indicated concentrations of PP2, SU6656, SKI I or SKI II. Cells were allowed to grow for at least two weeks, with agarose containing fresh inhibitor added to the wells every three to four days. Colonies were counted manually using a grid under 10 x magnification. The data presented are the mean of the number of colonies grown, relative to the control cells treated with DMSO, of three independent experiments done in duplicate. Standard deviation is given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the DMSO control is shown.

4.3.4.2. Effects of decreased Src, Fyn, or Yes expression on colony forming ability

The colony forming ability of HT29 and HepG2 cells with decreased Src, Fyn, or Yes expression was also examined. Although HT29 cells with decreased Src expression may have a reduced ability to form colonies in soft agar, the results of this assay were extremely variable, and no concrete conclusions can be drawn from this data (Figure 4.28 A).

In the case of HepG2 cells, transfection with siRNAs targeting Src resulted in a significant decrease in the number of colonies formed when compared to cells transfected with a scrambled control siRNA. Decreased expression of both Src and Yes in conjunction also inhibited HepG2 colony formation to the same extent as was observed for cells with decreased Src expression alone. In contrast, decreased Fyn or Yes expression did not significantly affect the ability of HepG2 cells to form colonies in soft agar (Figure 4.28 B).

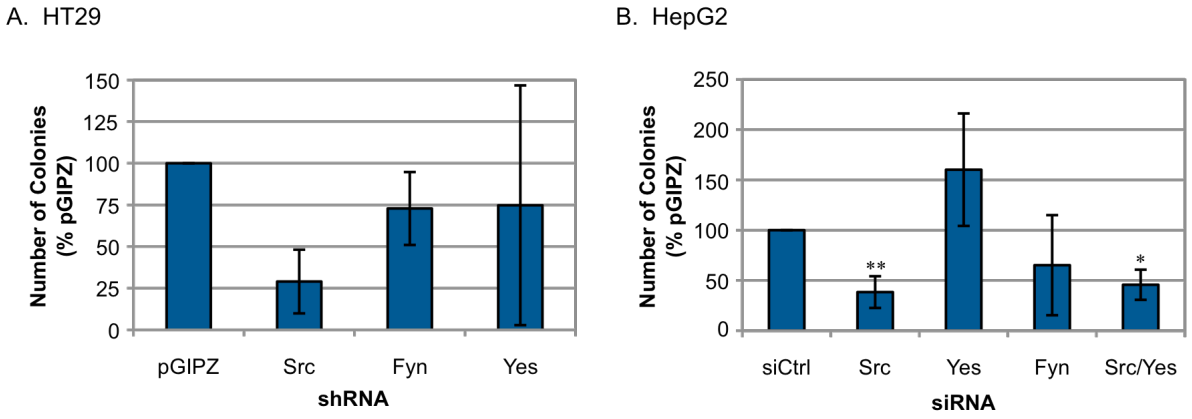


Figure 4.28. The colony forming ability of HT29 and HepG2 cells with decreased Src, Fyn, or Yes expression. A. HT29 cells stably expressing decreased levels of Src, Fyn or Yes, or **B.** HepG2 cells transfected with siRNAs targeting the Src family members were trypsinized, counted and 5000 cells seeded per well of a six well plate in agarose. Cells were allowed to grow for at least two weeks before colonies were counted manually using a grid under 10 x magnification. The data presented are the mean of the number of colonies grown, relative to the pGIPZ or siCtrl control cells, of five (HT29) or three (HepG2) independent experiments done in duplicate, with the standard deviation given as error. Statistical significance with a *P* value of less than 0.05 (*) or 0.01 (**) versus the pGIPZ or siCtrl control is shown.

4.3.4.3. Summary

Although the presence of at least two of the SFK inhibitors was sufficient to significantly inhibit the ability of both HT29 and HepG2 cells to form colonies in soft agar, the data obtained for HT29 cells with decreased expression of the individual SFKs was extremely variable and is not conclusive. In HepG2 cells, decreased expression of either Src alone, or Src and Yes together, resulted in fewer colonies than were formed from cells transfected with a scrambled control siRNA. Decreases in Fyn or Yes expression had no statistically significant effect on the colony forming ability of these cells (Table 4.5). In any case, the SFKs appear to play an important role in the colony forming ability of at least some cell lines.

Table 4.5. Summary of the effects of Src family kinase inhibitor treatment or decreased Src, Fyn, or Yes expression on the colony forming ability of selected cancer cell lines. No statistically significant change in colony forming ability following treatment or due to decreased protein expression is indicated by the – symbol; +++ indicates greater than 75% inhibition. Increased colony forming ability following treatment with the lower doses of inhibitors is indicated by an arrow. No conclusions can be drawn from assays examining the colony forming ability of HT29 cells with decreased Src, Fyn, or Yes expression due to large variability (n/a).

	shRNA/siRNA			Inhibitors			
	Src	Fyn	Yes	PP2	SU6656	SKI I	SKI II
HT29	n/a	n/a	n/a	↑/+++	+++	+++	–
HepG2	+++	–	–	+++	+++	–	–

5. DISCUSSION

The SFKs are overexpressed and activated in a number of human cancers, including those of the colon and breast, and contribute to cancer progression leading to metastasis through their involvement in a number of cellular phenotypes (Summy and Gallick, 2003). However, past studies have most commonly investigated the effects of Src expression alone on these phenotypes, or have inhibited multiple SFKs using SFK inhibitors. The goal of this study was therefore to examine the contributions of the individual SFKs to various cancer cell phenotypes, including proliferation, adhesion, migration, and colony forming ability, and to thereby identify if other SFKs besides Src itself are involved in these processes. In order to do this, two approaches were used. Namely, the kinase activity of multiple SFKs was inhibited by treating our cell lines of interest with four different commercially available SFK inhibitors, or the expression of individual SFKs was knocked down in these cell lines using either shRNA in HT29, HCT116, and SW480 cells, or siRNA in HepG2 cells, and the resulting phenotypic changes examined. This allowed the involvement of individual SFKs in selected cancer cell phenotypes to be assessed and compared to the cellular effects of inhibiting multiple SFKs.

5.1. Src family kinase expression and activity in cancer cells

The SFKs, which play important roles in various cancer cell phenotypes, are overexpressed in a wide variety of different cancers (Summy and Gallick, 2003). SFK mRNA expression was therefore investigated in a panel of colon cancer cell lines, while their protein expression was examined in the colon cancer cell lines, as well as in several breast cancer cell lines, lung cancer cell lines, the myeloid cell line U937, and the hepatocellular carcinoma cell line HepG2. It was found that Src, Yes, Fyn, Lyn, and Lck were all expressed at the mRNA and protein level in at least a subset of the cell lines investigated, and that the level of expression of a given SFK varied between cell lines (Hirsch *et al*., 2006). Furthermore, it was found that all of the cell lines investigated expressed at least one of the SFKs, and that different cell lines expressed different combinations of the family members. This was also found in a more recent study examining SFK expression in a panel of cell lines (Barraclough *et al*., 2007). Not surprisingly,

the other SFKs, namely Blk, Fgr, and Hck, which are generally thought to have expression restricted to cells of hematopoietic origin, were not observed (Hirsch *et al*., 2006).

Interestingly, while both Lyn and Lck are also generally regarded to be expressed mainly in hematopoietic cells, both of these proteins were expressed in the cell lines that we examined; Lyn in the majority of the cell lines, and Lck in a small subset of colon and lung cancer cell lines. This was likewise observed in another investigation of SFK expression in a large panel of colorectal cancer cell lines (Emaduddin *et al*., 2008). Lck has also previously been found to be expressed in some lung cancer cell lines (Veillette *et al*., 1987). As Lck is not generally expressed in normal epithelial tissue, its expression in certain cancers may indicate a role for this protein in epithelial cancers, as well as in hematopoietic cancers.

In addition to Src itself, other SFKs have also previously been found to have kinase activity in colon cancer cell lines (Park *et al*., 1993). Therefore, four cell lines representative of the combinations of SFKs expressed in the wider panel of cell lines were chosen for further study, and the kinase activity of the SFKs in these cells investigated using a commercially available tyrosine kinase assay kit. As this kit detects the total kinase activity of specifically immunoprecipitated proteins, it was possible to assess the activity of the individual SFKs. When HT29, HCT116, SW480, and HepG2 cells were examined for SFK kinase activity, not all of the SFKs expressed at the protein level within a given cell line were found to have significant kinase activity. However, the level of activity of those SFKs that were active roughly corresponded to the level of protein expression of that family member within a cell line. For instance, HT29 cells, with the highest level of Src protein observed, also had the highest level of Src kinase activity. In contrast, SW480 cells, which express a relatively lower level of Src protein, exhibited lower Src kinase activity. These findings support the idea that the high levels of Src activity observed in these cell lines are due to increased protein expression, which agrees with the findings that Src kinase activity correlates with the level of Src mRNA (Dehm *et al*., 2004) and protein (Iravani *et al*., 1998) expressed by a cell. Furthermore, different SFKs had kinase activity in different cell lines, and in addition to Src, both Yes and Fyn had detectable kinase activity in select cell lines. For instance, both Src and Yes kinase activity was observed in HT29 and HepG2 cells, while Src and Fyn activity was detected in the SW480 cell line.

In addition to SW480 cells, the kinase activity of the SFKs was also examined in SW620 cells. Although both cell lines showed Src and Fyn activity, with no Yes activity detected, the level of kinase activity of both Src and Fyn was increased in the SW620 cell line when compared to that of SW480 cells, whereby a moderate increase in Src activity and a greater increase in Fyn activity were observed. As the SW620 cell line is derived from a lymph node metastases from the tumour from which SW480 cells are derived, this supports the finding that Src activity increases with tumour progression (Talamonti *et al*., 1993). It also suggests that Fyn may also be involved in colon cancer progression, at least in a subset of colon cancers. Indeed, it has been reported that there is more SFK activity in a number of colon cancer cell lines than can be accounted for by the level of kinase activity of Src itself, suggesting that other family members may also have significant kinase activity in these cells (Emaduddin *et al*., 2008). As Src, Fyn, and Yes are all expressed and exhibit kinase activity in at least some of the colon cancer cell lines investigated, it is possible that all three of these proteins are involved in at least some cancer cell phenotypes in certain cell lines.

It was of interest that Lyn was expressed at the protein level in the majority of the cell lines investigated, as this SFK has traditionally been thought to be mainly expressed in hematopoietic cells. However, Lyn has previously been found to be expressed at the mRNA level in lung tissue (Holtrich *et al*., 1991), as well as in cultured airway smooth muscle cells (Pertel *et al*., 2006), and human bronchial epithelial cells (Zhao *et al*., 2006). Despite having been reported to be expressed in the lung, the involvement of Lyn in lung cancers has not been extensively studied. In contrast, Src has been found to be highly expressed at the protein level in lung cancers (Mazurenko *et al*., 1992), and is significantly activated in NSCLCs, where the level of Src activity is correlated with tumour size (Masaki *et al*., 2003). The kinase activity of both Lyn and Src was therefore investigated in selected lung cancer cell lines. Although all of the cell lines examined exhibited Src activity, Lyn activity was detected only in the two cell lines with the highest Lyn protein expression, the level of which was proportional to the level of Lyn protein expressed in these cell lines. When a tissue microarray containing samples of various types of lung cancer was examined, it was found that although Lyn was expressed in the majority of the samples, in most cases the level of expression was low. However, the majority of the squamous cell carcinomas, large cell carcinomas, and atypical carcinoids represented on the slide showed high levels of Lyn protein expression in the majority of the

cells in the sample. This suggests that Lyn expression and activity may be significant in some histological types of lung cancer but not others, and that Lyn, as well as Src, may contribute to the cancerous phenotypes of certain lung cancers.

5.1.4. Summary

Our work has demonstrated that although all of the cancer cell lines investigated expressed multiple SFKs, the particular SFKs that are expressed and active in a given cell line vary significantly. Furthermore, not all of the SFKs expressed in a given cell line necessarily have significant kinase activity, at least under our assay conditions. For instance, while Src was found to have kinase activity in all of the cell lines investigated, both Fyn and Yes had detectable kinase activity in only a subset of the colon cancer cell lines, while Lyn kinase activity was detected in a subset of the lung cancer cell lines. This suggests that more than one SFK may be involved in the cancer phenotypes of these cells. Lyn in particular may have a role in lung cancer, as it was found to be expressed in many of the lung cancer cases present on a tissue microarray stained for Lyn protein expression. Furthermore, as the squamous cell carcinomas, large cell carcinomas, and atypical carcinoids represented on the slide showed relatively high levels of Lyn protein expression in some of the cells in the sample, Lyn may be involved in certain histological types of lung cancer in particular. Although we did not investigate the roles of the SFKs in normal colon cells, our findings point to the potential involvement of a number of the SFKs in different cancers, and indicate that the particular SFKs involved may vary between cell lines.

5.2. RNA interference-mediated silencing of the Src family kinases

In order to examine the involvement of the individual SFKs in cancer cell phenotypes it was necessary to be able to target these proteins individually, without affecting the expression or activity of the other family members. As the SFK inhibitors currently available inhibit multiple SFKs (Hanke *et al*., 1996; Liu *et al*., 1999; Blake *et al*., 2000), a sequence based RNAi approach was used to target these proteins, which allowed the expression of a single SFK to be knocked down without affecting the expression of the other SFKs. Both chemically synthesized siRNA and a vector based shRNA approach were used.

The siRNA approach involves the introduction of 21 nucleotide long, chemically synthesized siRNAs into cells using a lipid based transfection reagent. For four hours following transfection, siRNAs accumulate in the cytoplasm in both an intact and disassociated form (Rao *et al*., 2009). Once in the cytoplasm, the siRNAs direct the sequence-based degradation of the complementary mRNA; this activity reaches a peak at around 24 hours following introduction into the cell. However, as the majority of the siRNAs transfected into a cell will have been degraded by 48 hours following transfection, siRNA produces only a temporary silencing of the targeted protein (Rao *et al*., 2009), the length of which is related to the rate of cell division (Dykxhoorn *et al*., 2003). The level of silencing elicited by transfected siRNAs also depends greatly on transfection efficiency, which can be variable, particularly in difficult to transfect cell lines. Therefore, large variations in the level of silencing may occur, both between experiments and cell lines (Amarzguioui *et al*., 2005). In our experiments, the transfection of HepG2 cells with siRNAs targeting the SFKs resulted in a significantly higher level of knockdown than was achieved in HT29 cells. The level of knockdown in HT29, HCT116, and SW480 cells also varied greatly between replicates (data not shown). Therefore, although the siRNA approach was initially used to silence the expression of the SFKs both individually and in combination in all four of our cell lines of interest, it was preferable to use the vector based shRNA approach in order to decrease the expression levels of the SFKs in a stable manner.

In contrast to the transient knockdown of protein expression observed following the transfection of siRNAs into cells, stable knockdown can be achieved by using a vector based approach. In this method, sequences coding for shRNAs are cloned into an expression vector (in our case, of lentiviral origin) and introduced into target cells. This results in the production of the shRNA by the cell itself, as it is transcribed by either RNA polymerase II or III (Rao *et al*., 2009). The transcript produced contains a hairpin stem-loop structure that can be processed in the nucleus to produce individual shRNAs with a two-nucleotide 3' overhang. Following transport to the cytoplasm, the hairpin loop is cleaved, forming a double-stranded siRNA that mediates the degradation of the target mRNA. As the shRNAs are synthesized by the cell into which it has been introduced, a stable knockdown is achieved. Indeed, fewer than five copies of an shRNA vector are sufficient to result in the continuous knockdown of a target protein (Rao *et al*., 2009). Vectors containing an shRNA sequence targeting Src, Fyn, or Yes, as well

as an empty control vector, were therefore introduced into our cell lines of interest in order to stably decrease the expression levels of these proteins.

Although HT29, HCT116, and SW480 cells with stable knockdown of Src, Fyn, or Yes were successfully produced, the shRNA approach was unsuccessful in generating HepG2 cells with stable knockdown of the SFKs. However, it was possible to examine the effects of decreased SFK expression in HepG2 cells using siRNA, as the transfection of this cell line consistently resulted in a significant level of knockdown. Although there was a near complete siRNA-mediated knockdown of Src in these cells for over 144 hours post transfection, the Yes siRNA was not as efficient, and resulted in less significant knockdown. However, Yes levels also remained decreased for over 144 hours. Although the HepG2 colony forming assays were performed over two weeks, this length of knockdown allowed differences in colony forming ability to be observed. Results obtained from siRNA knockdown in the other cell lines produced extremely variable levels of knockdown between experiments, and are therefore not presented in this work (data not shown).

Both siRNA and shRNA can result in off-target effects, whereby they cause effects other than those due to the degradation of the target mRNA. These effects are sequence related, as RNAi mediated by the same sequence in both siRNA and shRNA protocols results in the same off-target effects in multiple cell lines (Jackson *et al*., 2006). For instance, partial sequence complementarity of the siRNA sequence to mRNAs other than the target, or partial complementarity to the guide strand of the RNAi construct, can result in the suppression of genes other than the intended gene (Rao *et al*., 2009). The severity of off-target effects appears to be unrelated to the ability of an siRNA to silence its target, however (Jackson *et al*., 2003). Other non-specific effects on cellular behaviour may occur due to the delivery of chemically synthesized siRNAs or vectors into a cell, or due to the expression of the shRNA construct itself (Rao *et al*., 2009).

In addition to having different off-target effects, the efficiency at which different siRNA and shRNA sequences are able to silence the expression of a single protein also varies greatly. This can depend on the sequence used, as not all sequences to a given target are able to silence the expression of a protein to the same degree (Dykxhoorn *et al*., 2003). For this reason, multiple siRNA and shRNA sequences targeting each SFK were tested for the ability to silence their targets. Ideally, at least two of these sequences would have resulted in the stable knockdown of

each SFK, in order to control for any non-specific effects exerted by a given sequence. However, only one of the shRNA sequences examined for each protein produced a significant level of knockdown. All further experiments were therefore carried out using the single most effective siRNA or shRNA sequence to decrease the expression of Src, Fyn, or Yes.

Unfortunately, complete knockdown of the SFKs was not achieved even when using the most effective shRNA sequence to target each SFK, and, in some cases, was very poor. The level of knockdown achieved following the introduction of Src, Fyn, or Yes shRNA vectors into HT29, HCT116, and SW480 cells was assessed quantitatively using the Odyssey software (Li-Cor), which determines the intensity of protein bands on Western blots. The level of Src protein was decreased significantly in all of the cell lines investigated, with knockdown from 70% to 80%; however, the knockdown of Fyn and Yes was less effective. Although Fyn expression was decreased by 70% in HCT116 cells, the level of Fyn knockdown was closer to 60% in both HT29 and SW480 cells. The expression of Yes in particular proved to be difficult to knock down using both shRNA and siRNA; Yes levels were decreased by only 55% to 60% in the stable cell lines created. While it is possible that the remaining protein was sufficient to mask any involvement of these proteins in the cellular processes investigated in this work, the level of knockdown achieved should have allowed differences between the phenotypes of parental cells and those with decreased SFK expression to be observed.

5.2.4. Summary

In order to inhibit the protein expression of individual SFKs within a cell line, an RNAi approach was used to decrease the expression of Src, Fyn, or Yes in HT29, HCT116, SW480, and HepG2 cells. While siRNA was used to transiently reduce SFK expression in HepG2 cells, an shRNA approach was used to achieve stable knockdown of the SFKs in HT29, HCT116, and SW480 cells. Although multiple siRNA and shRNA sequences were initially tested for their ability to reduce SFK expression, only one sequence for each of Src, Fyn, and Yes was found to result in significant knockdown. Had more than one sequence targeting each SFK resulted in successful knockdown the presence of off-target effects could have been controlled for to some degree; however, as only one sequence effectively knocked down each SFK it is possible that off-target effects may have affected the results of our assays. Cell lines in which different combinations of SFKs were knocked down would have also been useful in

determining if the SFKs are able to compensate for a loss of expression of one of the family members in given cellular functions. However, as all of the vectors used contained the same selective marker, the knockdown of multiple SFKs in single cells was not achieved. Therefore, further experiments examined the effects of decreasing the expression of only a single SFK on selected cellular phenotypes.

As SFK knockdown was not complete in the stable cell lines produced, the effects of decreased Src, Fyn, or Yes expression on the cellular processes investigated in this work are likely not as pronounced as if complete knockdown had been achieved. In particular, Fyn and Yes were more resistant to knockdown than was Src, despite a number of sequences being assessed for their ability to decrease the expression of these proteins. Between 20% and 30% of normal Src levels remained even in the cells expressing shRNAs targeting Src, while those in which Fyn or Yes were knocked down expressed up to 40% of the normal levels of these proteins. As this was the case, the level of Src, Fyn, or Yes protein remaining in a cell following incomplete knockdown could be sufficient to carry out the normal cellular functions of these proteins, thereby masking their involvement in given cellular phenotypes. However, while the differences in cellular behaviour between the control cells and those with decreased SFK expression may have been less significant than would have been observed had complete knockdown occurred, the level of knockdown achieved likely allowed some of the major differences in proliferation, adhesion, migration, and colony formation to be detected.

5.3. Involvement of Src family kinases in cancer cell phenotypes

A large number of studies have investigated the involvement of the SFKs in various cancer cell phenotypes, and changes in the expression and activity of these proteins have been implicated in altered cellular adhesion, apoptosis, angiogenesis, tumour growth, and metastasis (reviewed in Irby and Yeatman, 2000). However, many of these studies have only used a single chemical SFK inhibitor to examine the involvement of the SFKs in various cellular phenotypes. As chemical inhibitors may have significant off-target effects, four different SFK inhibitors were used in the studies described herein so that their effects could be compared. Furthermore, RNAi was used to specifically decrease the expression of Src, Fyn, or Yes in selected cell lines in order to examine the involvement of individual SFKs in our selected cellular phenotypes. Examining the phenotypic changes of cells in which the expression of a

single SFK was decreased in conjunction with those of cells in which the kinase activity of all of the SFKs was inhibited was undertaken in order to provide a clearer picture of the actual involvement of these proteins in selected cancer cell phenotypes.

5.3.1. Cellular Proliferation

In order to examine the involvement of the SFKs in the cellular proliferation of HT29, HCT116, SW480, and HepG2 cells, the effects of either SFK inhibition or decreases in the expression level of individual SFKs were examined. To assess the proliferation of our cell lines of interest, two different approaches were undertaken. A method that is commonly used to assess cellular proliferation is the use of tetrazolium salts, which gives an indication of the number of metabolically active cells in a sample (Mosmann, 1983; Cory *et al*., 1991). This method can be used to determine cell numbers, as actively proliferating cells will reduce tetrazolium salts, such as MTS and 3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to a formazan product that can be measured colourimetrically. As the amount of formazan formed correlates directly with the number of metabolically active cells present in a culture, this assay can be used to monitor cellular proliferation. There are, however, disadvantages to this technique. For instance, the use of tetrazolium salts gives a measure of the number of only healthy, metabolically active, cells within a population. MTS assays are also not as sensitive in samples with few cells (Huang *et al*., 2004). Since this is the case, small differences in the number of cells seeded may significantly impact results. In our investigations, this may have prevented an effect on proliferation from being observed since, as the populations of knockdown cell lines were counted separately before seeding, there were likely differences in the number of cells seeded. Although the MTS assay did not show significant decreases in proliferation, HT29 cells with decreased Src or Yes expression were observed qualitatively to grow more slowly than either the parental cell line or the cells transduced with the pGIPZ control vector. Therefore, it was necessary to manually count the numbers of cells present within a larger sample in order to examine the proliferation of cells with decreased SFK expression. Unlike assays using tetrazolium salts, counting cells is a measure of the number of all of the cells in a sample, and is therefore a direct measure of cellular proliferation. The colourimetric MTS assay was therefore used in conjunction with

counting cells in order to examine the effect of decreased SFK expression or activity on cellular proliferation.

By examining four cell lines in conjunction, it was possible to identify differences in the involvement of the SFKs in cellular proliferation between cell lines. Indeed, our findings suggest that the effects of SFK inhibition on proliferation are both cell line and inhibitor specific. For instance, PP2 treatment significantly inhibited the proliferation of only HT29 and HCT116 cells, and did not affect that of SW480 or HepG2 cells. Furthermore, HCT116 cells were more susceptible to the SFK inhibitors than the other cell lines, as PP2, SU6656, and SKI II all reduced the proliferation of this cell line. Conversely, the SFK inhibitors had no effect on the proliferation of SW480 and HepG2 cells. This suggests that SFK inhibition affects proliferation in a cell line-specific manner. Although this was the case in our findings, many of the studies that have used SFK inhibitors to inhibit the activity of the SFKs in a variety of cell lines have found that SFK inhibition results in decreased cellular proliferation. For instance, the treatment of CML (Golas *et al*., 2003), osteosarcoma (Spreafico *et al*., 2008), pancreatic (Nagaraj *et al*. 2010), and breast cancer cell lines (Moasser *et al*., 1999) with SFK inhibitors has been shown to inhibit the proliferation of these cells. Many of the investigations that have used PP2 to inhibit the SFKs have also reported effects on proliferation: treatment with this inhibitor has been found to both inhibit the proliferation and increase the apoptosis of a medulary thyroid cancer cell line (Liu *et al*., 2004), as well as to inhibit the anchorage-dependent and independent growth of three different breast cancer cell lines (Ishizawar *et al*., 2004). Finally, in an investigation of a number of colorectal cancer cell lines, it was found that all of the sixteen cell lines investigated were sensitive to both PP2 and SU6656 treatment, whereby proliferation was inhibited in a concentration dependent manner (Emaduddin *et al*., 2008). In contrast, a further study investigating the proliferation of several colon cancer cell lines following treatment with the clinically relevant SFK inhibitor dasatinib found that this inhibitor only inhibited the proliferation of two out of the twelve cell lines investigated, and had no effect on the proliferation of the other ten cell lines (Serrels *et al*., 2006). Taken together, these studies, as well as our own, suggest that the effects of SFK inhibitors on cellular proliferation are variable.

In addition to differences in susceptibility to SFK inhibitors between cell lines, differences in the effects of SFK inhibitor treatment on proliferation observed between studies could be due

to incomplete inhibition of the SFKs at lower concentrations, or because of increased non-specific effects due to higher concentrations of inhibitor. That at least some of the effects of these inhibitors on proliferation are due to non-specific effects is suggested by our finding that not all of the SFK inhibitors affected proliferation in a given cell line. For instance, the proliferation of HT29 cells was inhibited by PP2 and SKI I, while treatment with SU6656 and SKI II had no effect. Similarly, the proliferation of HCT116 cells was not affected by treatment with SKI I, but was inhibited to some extent by the other three SFK inhibitors. If the effects of these drugs on proliferation were due solely to the inhibition of the SFKs, it would be expected that all of the SFK inhibitors would affect proliferation similarly. However, the large number of off-target effects that these inhibitors may elicit could result in either increased or decreased proliferation, or in no effect on proliferation being observed. Taken together, our results suggest that the effects of SFK inhibitors on the proliferation of various cell lines are complex, and likely depend on both cell line and the inhibitor being used.

In addition to SFK inhibition, the proliferation of cells expressing decreased levels of Src, Fyn, or Yes was examined. As was found when the SFKs were inhibited, the effects of decreasing the expression of the individual SFKs on proliferation appear to depend upon cell line. For instance, decreased Src expression inhibited the proliferation of HT29 and SW480 cells, but had no effect on the proliferation of HCT116 or HepG2 cells. By examining the effects of decreasing the expression of all three of the ubiquitously expressed SFKs individually in our study, we also demonstrated that not only Src, but also Yes, and to a lesser extent, Fyn, likely play a role in cellular proliferation. For instance, the proliferation of HT29 cells was equally inhibited in cells with decreased Src or Yes protein levels. HT29 cells with decreased Fyn levels were also found to proliferate more slowly than the control cells, although not to the extent of the cells in which either Src or Yes were knocked down. This suggests that, at least in this cell line, all three of the ubiquitously expressed SFKs are involved in regulating proliferation. This corresponds to the more dramatic decrease in proliferation observed when HT29 cells were treated with SFK inhibitors, which inhibit the kinase activity of multiple SFKs. In contrast, the proliferation of HCT116 cells was not affected by decreased levels of Src, Yes, or Fyn, as measured by either an MTS assay or in direct cell counts. As HCT116 proliferation was inhibited by SFK inhibitor treatment, this may indicate that multiple SFKs must be targeted in order for an effect on proliferation to be observed in some cell lines. As

there is evidence that the SFKs are able to compensate for a loss in activity of one or more family members, this could be the case (reviewed in Lowell and Soriano, 1996). Interestingly, although the proliferation of SW480 cells was not significantly affected by SFK inhibitor treatment, a growth curve revealed that SW480 cells with decreased Src expression had significantly slower proliferation than the control cells. Although SW480 cells with decreased Fyn expression also had significantly decreased proliferation when compared to either untransduced control cells or those that had been transduced with an shRNA targeting Yes, the proliferation of these cells was not significantly different than that of the control pGIPZ cells, which had a reproducible decrease in proliferation when compared to the control parental cells. The involvement of Fyn in the proliferation of SW480 cells therefore remains inconclusive. It appears that SFK activity is not necessary for the proliferation of HepG2 cells, as no change in proliferation was observed upon treatment with SFK inhibitors, or when Src or Yes protein levels were decreased either individually or in concert. Therefore, our results suggest that the involvement of the SFKs in cellular proliferation is cell line specific, and that more than one SFK may be involved in this process within a given cell line.

The involvement of the SFKs in the proliferation of cancer cell lines has previously been investigated by varying the expression levels of these proteins, either through the use of siRNA or antisense, or through overexpression. For instance, HT29 cells in which Src has been silenced through the use of antisense have decreased proliferation (Staley *et al*., 1997), which corresponds to our findings in this cell line. Similarly, siRNA mediated decreases in Src expression have been found to result in the reduced proliferation of MCF7 breast cancer cells (González *et al*., 2006). It has also been shown in primary CML blasts, as well as in myeloid cell lines, that there is a decrease in cellular proliferation coupled with an increase in apoptosis when Lyn is specifically silenced using siRNA (Ptasznik *et al*., 2004). However, Src antisense was found to have no effect on the proliferation of an ovarian cancer cell line (Wiener *et al*., 1999), thereby supporting the idea that the SFKs affect cellular proliferation in a cell line specific manner. In contrast to most findings obtained by decreasing SFK expression, the overexpression of Src has been found to have little effect on proliferation. For instance, no effects on proliferation were observed in response to elevated Src kinase activity in KM12C, HCT116, or SW480 cells, either *in vitro*, or when injected into mice (Jones *et al*., 2002; Welman *et al*., 2006). Similarly, the proliferation of colon cancer cell lines was not affected by

the overexpression of Yes, either *in vitro* or *in vivo* (Barraclough *et al*., 2007). However, the finding that increased SFK protein levels and activity do not have a significant effect on cellular proliferation may simply indicate that the cells being assayed already express the SFKs at a level that results in the maximum amount of proliferation signalling being achieved; in this case, having surplus amounts of the SFKs would not result in an effect on proliferation. The disparate findings of investigations examining the effects of SFK activity on cellular proliferation suggest that the SFKs may be involved in the proliferation of some cell lines and not others. Our results also support the idea that the effects of the SFKs on proliferation are cell line specific, as both treatment with SFK inhibitors and decreases in SFK expression inhibited the proliferation of only a subset of the cell lines investigated.

5.3.2. Cellular Adhesion

In addition to cellular proliferation, the SFKs have also been implicated in a variety of signalling pathways involved in cellular adhesion to both the extracellular matrix (ECM) and to other cells. The adhesion of cells to the ECM is important to the growth of most non-cancerous cells, as it not only provides support to the cells embedded within it, but also influences the growth, adhesion, and motility of these cells (reviewed in Frantz *et al*., 2010). The ECM is composed primarily of polysaccharides and fibrous proteins that have been secreted by nearby cells and organized into a network. The major scaffolding protein of the ECM is collagen, which is involved in stabilizing, strengthening, and organizing other components of the ECM (Rozario and DeSimone, 2010). Although there are a number of different collagens, Type I collagen is the most abundant (Di Lullo *et al*., 2002). Fibronectin, in contrast, is a large glycoprotein that is involved in cellular adhesion and migration, as well as in organizing the ECM, through its interactions with integrins and collagen (reviewed in Pankov and Yamada, 2002; Singh *et al*., 2010). The ability of our cell lines of interest to adhere to both collagen I and fibronectin following treatment with SFK inhibitors or decreases in Src, Fyn, or Yes expression was investigated.

Our results indicate that although SFK inhibition was able to decrease the level of cellular adhesion to fibronectin and collagen I, this was dependent upon the inhibitor being used, the concentration of the inhibitor, and the cell line being investigated. Although PP2 and SKI I inhibited the adhesion of the majority of the cell lines examined to both fibronectin and

collagen I, the extent of this inhibition was cell line specific. For instance, the adhesion of HT29 cells to fibronectin was inhibited to the greatest extent, with greater than 50% inhibition by three of the four SFK inhibitors investigated. The adhesion of HepG2 cells to fibronectin was also significantly reduced by the majority of the inhibitors. In contrast, none of the inhibitors decreased the adhesion of HCT116 cells to fibronectin, while only PP2 inhibited the adhesion of SW480 cells. Furthermore, although the treatment of HT29 cells with SU6656 resulted in a decrease in adhesion to both fibronectin and collagen I of more than 50%, SU6656 and SKI II generally had much weaker effects on the adhesion of the cell lines examined. This clearly demonstrates that the effects of the different SFK inhibitors on adhesion are variable.

The effect of SFK inhibition on adhesion also depended upon the adhesion molecule being assayed, as the adhesion of our cell lines of interest to both fibronectin and collagen was affected to different extents by the SFK inhibitors, with the exception of HT29 cells. For instance, the adhesion of HepG2 cells to fibronectin in the presence of PP2 was decreased by approximately 40% while their adhesion to collagen I was inhibited by only 20%. In contrast, none of the inhibitors tested impacted the adhesion of HCT116 cells to fibronectin, although PP2 treatment reduced the adhesion of these cells to collagen following PP2 treatment by 40%. The adhesion of this cell line to collagen I was also inhibited by the presence of SKI I and SKI II by 25%. That the SFKs are involved in cellular adhesion to different components of the ECM to different extents has previously been suggested by the finding that although the adhesion of *src*$^{-/-}$ cells to fibronectin was reduced when compared to Src expressing cells, no difference in the adhesion of these cells to collagen was observed (Kaplan *et al*., 1995). This indicates that the SFKs may be involved in cellular adhesion to different components of the ECM. Furthermore, our results suggest that this may be cell line dependent.

Interestingly, all of the inhibitors examined, with the exception of PP2, caused a dose-dependant increase in the adhesion of at least one cell line to either fibronectin, collagen I, or both. Interestingly, this increase in adhesion was observed only when cells were treated with 2 μM the of the inhibitors. This was particularly evident with SKI II, as treatment with this inhibitor increased the adhesion of all of the cells lines investigated to collagen I and three of the four cell lines to fibronectin. In contrast, treatment with low concentrations of SU6656 and SKI I resulted in an increase in adhesion of only a subset of the cell lines. Despite the increase in cellular adhesion when the cells were exposed to low doses of inhibitor, higher

concentrations of these inhibitors either did not statistically impact adhesion to either fibronectin or collagen I, or resulted in a decrease in adhesion. One possibility for this is that these inhibitors may have unexpected effects on other proteins at lower concentrations that result in an increase in adhesion. The possible incomplete inhibition of the SFKs at lower doses of inhibitor may also be a factor. As this increase in adhesion was observed following treatment with most of the inhibitors used in these studies, it may indicate that SFK inhibitors elicit different effects upon their targets depending upon the concentration used, which may be an important consideration when using these drugs as anti-cancer agents.

Despite the ability of the SFK inhibitors to inhibit cellular adhesion to both fibronectin and collagen I, only HT29 and HepG2 cells with decreased Src and Yes expression were observed to have reduced adhesion to these molecules. Furthermore, decreases in Src and Yes expression only inhibited the adhesion of HT29 cells to fibronectin; no effect on the adhesion of these cells to collagen I was observed. The adhesion of HepG2 cells to fibronectin was also reduced in response to decreases in Src and Yes expression, while decreased Yes expression also reduced the adhesion of these cells to collagen I. In contrast, HCT116 and SW480 cells with decreased SFK expression did not show any difference in adhesion to either fibronectin or collagen I, suggesting that more than one of the SFKs may be involved in adhesion in these cells, and that the loss of a family member can be compensated for by the remaining SFKs. If this were the case, the loss of an individual SFK would not result in any observable change in adhesion. This could also explain the finding that, although the adhesion of *src*$^{-/-}$ fibroblasts to a fibronectin matrix is inhibited somewhat, these cells retain the ability to adhere, suggesting that although Src may be required for maximum adhesion, it is not necessary for adhesion to occur (Kaplan *et al*., 1995). As *src*$^{-/-}$ cells express other SFKs this could be due to compensation by other family members. Our finding that treatment with all of the SFK inhibitors reduced the adhesion of at least a subset of the cell lines examined to a greater extent than was observed when single SFKs were knocked down also suggests that the SFKs are able to compensate for the loss of activity of other family members. Indeed, the finding that the knockdown of individual SFKs did not affect the adhesion of two of the cell lines investigated in this work to either fibronectin or collagen I, while SFK inhibition significantly impacted their adhesion to both of these adhesion molecules, suggests that multiple SFKs are involved in

this process. However, it is also possible that the level of knockdown of the SFKs achieved in this work was not sufficient to observe effects on cellular adhesion.

In agreement with our findings, the SFKs have previously been shown to be involved in cellular adhesion to the ECM. For example, while elevated Src expression has been found to result in the increased adhesion of KM12C colon cancer cells to fibronectin, this adhesion was inhibited by treatment with either PP2 or SU6656 (Jones *et al*., 2002). Furthermore, the expression of a kinase-deficient, dominant-negative Src prevented KM12C cells from extending and retracting protrusions required for adhesion to collagen (Brunton *et al*., 2005). Indeed, the major effect of abnormal Src signalling in the KM12C colon cancer cell line has been found to be on both cell-cell and cell-ECM adhesion, rather than on proliferation (Avizienyte *et al*., 2002; Jones *et al*., 2002; Brunton *et al*., 2005). The expression of a dominant-negative Src has also been shown to result in a significant reduction in the adhesion of MCF7 breast cancer cells to fibronectin (González *et al*., 2005), while the adhesion of prostate cancer cells to fibronectin was inhibited by the SFK inhibitor dasatinib (Nam *et al*., 2005). These studies, along with our work, demonstrate that the SFKs are involved in the adhesion of cancer cell lines to components of the ECM.

5.3.3. Cellular Migration

The SFKs have strongly been implicated in cellular migration, as the loss of their activity either due to SFK inhibitor treatment or through decreased expression has been found to result in a reduced ability of cells to migrate. In agreement with previous studies, we found that the migration of all three of the cell lines examined was significantly reduced by treatment with both PP2 and SKI I. Furthermore, we demonstrated that the level of inhibition depended upon both the cell line and inhibitor being investigated. For instance, PP2 treatment inhibited the migration of all of the cell lines to the greatest extent, with the migration of both HT29 and SW480 cells being completely inhibited, and that of HepG2 cells being decreased by 70%. Similarly, PP2 treatment has been reported in the literature to result in the decreased motility of osteosarcoma cells (Azuma *et al*., 2005), prostate carcinoma cell lines (Slack *et al*., 2001), and a murine melanoma cell line with high metastatic potential in which Fyn is selectively activated of the SFKs (Huang *et al*., 2003). Similar results have been found with other SFK inhibitors as well, including the clinically relevant SKI-606 and dasatinib; SKI-606 has been found to inhibit

the motility and invasion of breast cancer cell lines (Vultur *et al*., 2008), while dasatinib has been shown to inhibit the migration of melanoma cells (Buettner *et al*., 2008), prostate cancer cell lines (Nam *et al*., 2005), and malignant pleural mesothelioma cells (Tsao *et al*., 2007), as well as several human sarcoma cell lines from both bone and soft tissue (Shor *et al*., 2007). Taken together, these studies strongly implicate the SFKs in cellular migration.

In addition to investigating the effects of inhibiting all of the SFKs on cellular migration, the effects of decreased Src, Fyn, and Yes expression were also examined. Our investigations found that not only are several of the SFKs involved in cellular migration, but that more than one SFK may be involved in this process in a given cell line. For instance, HT29 cells with decreased expression of Src, Yes, and Fyn all had reduced migration when compared to control cells; the migration of HT29 cells with decreased Src and Fyn expression was inhibited by 30%, while that of cells with decreased Yes expression was inhibited by 45%, when compared to the pGIPZ control cells. This suggests that all three of the ubiquitously expressed SFKs may play a role in migration in this cell line. However, when shRNA expressing SW480 cells were examined for changes in cellular migration, only the migration of cells with decreased Src expression was inhibited, while no effect was observed in SW480 cells with decreased Fyn expression. This was surprising, as Fyn, which has been implicated in cellular migration in both cancer and normal cells, was found to have kinase activity in SW480 cells. In contrast, no statistically significant effect was observed on the migration of HepG2 cells when Src, Yes, or Fyn were knocked down, or when Src and Yes were knocked down simultaneously. This again suggests that the involvement of the individual SFKs in migration is cell line specific. Further evidence supporting this idea comes from a study that found that when cells lacking Src, Yes, and Fyn were reconstituted with Src, Yes, or Fyn individually, only Src was required for villin-induced regulation of migration (Mathew *et al*., 2008), despite other findings implicating Yes and Fyn in the migration of various other cell lines (Mariotti *et al*., 2001; Huang *et al*., 2003; Azuma *et al*., 2005; Barraclough *et al*., 2007).

A number of the SFKs have previously been implicated in cellular migration by studies that have varied their level of expression. For instance, both Src siRNA and the expression of a dominant-negative Src have been shown to inhibit the migration of MCF7 breast cancer cells (González *et al*., 2006). The motility of KYN-2 hepatocellular carcinoma cells, which have high levels of Src activity and are normally highly motile, has likewise been shown to be

inhibited by the expression of a dominant-negative Src (Sakamoto *et al*., 2001). Furthermore, siRNA-mediated decreases in the expression of both Src and Fyn have been found to inhibit the EGFR-dependent motility of glioblastoma cells (Lu *et al*., 2009). Of the SFKs, Fyn in particular has often been implicated in cellular migration. For instance, the expression of a dominant-negative form of Fyn in squamous carcinoma cells was shown to result in a decrease in the ability of these cells to migrate both *in vivo* and *in vitro* (Mariotti *et al*., 2001). A further study found that the upregulation of Fyn, but not other SFKs, corresponded to an increase in cell motility and cell spreading in a murine melanoma cell line with high metastatic potential (Huang *et al*., 2003). Similarly, the overexpression of Fyn in a subline of HuO9 osteosarcoma cells with low metastatic potential has been shown to result in increased cell motility (Azuma *et al*., 2005). In contrast, increased Fyn activity did not have a significant effect on the migration of glioblastoma cells (Stettner *et al*., 2005), although Lyn siRNA was able to significantly inhibit the migration of these cells (Ding *et al*., 2003). Yes has also been implicated in migration, as the expression of an active Yes in LS174T colon cancer cells resulted in the increased motility of these cells (Barraclough *et al*., 2007). Taken together, these studies indicate that multiple SFKs are involved in cellular migration, at least in some cell types, which corresponds to our finding that an RNAi-mediated decrease in Src, Fyn, or Yes expression is capable of inhibiting the migration of certain cell lines. That the migration of these cells was not reduced to the same extent as it was when multiple SFKs were inhibited could be either due to the incomplete knockdown of the SFKs, or could indicate that multiple SFKs need to be targeted in order for the full involvement of these proteins in migration to be observed. As we found that the knockdown of Src, Fyn, and Yes in HT29 cells all decreased migration this may be the case.

5.3.4. Colony Forming Ability

In addition to examining the effects of decreased SFK expression and activity on the cellular proliferation, adhesion, and migration of our cell lines of interest, the colony forming ability of HT29 and HepG2 cells was also assessed, as these two cell lines were found to form significant numbers of colonies. Since cells must have certain characteristics of transformed cells in order to grow in a soft agar matrix, the ability of cells to form colonies is viewed as a measure of transformation. For instance, they must be able to grow with a lack of contact inhibition and in

the absence of anchorage (Hanahan and Weinberg, 2000). As cellular motility is impeded when cells are grown in soft agar, single cells with a transformed phenotype will form colonies, which may be counted in order to assess the colony forming ability of a given cell line.

When HT29 and HepG2 cells were examined for colony forming ability in the presence of SFK inhibitors it was again found that the effects observed were inhibitor, concentration, and cell line specific. For instance, PP2 and SU6656 were able to inhibit the colony formation of both cell lines, while SKI I was able to prevent HT29 cells, but not HepG2 cells, from forming colonies. Similarly, both SU6656 and SKI-606 have previously been found to prevent HT29 cells from forming colonies in soft agar (Laird *et al*., 2003; Golas *et al*., 2005).

When the ability of HT29 and HepG2 cells with decreased Src, Fyn, and Yes expression to form colonies was examined, it was observed that the colony formation of HepG2 cells with decreased Src expression was significantly inhibited. In contrast, although cells with decreased Yes expression were observed to form larger colonies than the control cells, the number of colonies formed was not affected significantly. It is, however, possible that the involvement of Yes in this process may have been masked by the incomplete knockdown of Yes in these cells. The colony forming ability of HepG2 cells with decreased expression of both Src and Yes together was inhibited to the same extent as cells with decreased Src expression alone. Although it appeared that decreased Src expression may also reduce the colony forming ability of HT29 cells, the number of colonies formed from this cell line was extremely variable between experiments, and no conclusive conclusions may be drawn from these results. This was likely due to differences in seeding, as each cell line with decreased Src, Fyn, or Yes expression needed to be counted and seeded separately. In contrast, cells were seeded from a single dilution in the experiments examining the effects of SFK inhibitor treatment, thereby increasing the likelihood that an identical number of cells was seeded in every well. However, the observation that decreased Src expression in HepG2 cells, and potentially HT29 cells, impedes colony formation agrees with the finding that an siRNA-mediated reduction in Src expression decreased the ability of four breast cancer cell lines and two colon cancer cell lines, including HT29, to form colonies in soft agar (Zheng *et al*., 2008). Taken together, these results suggest that SFK expression is required for cells to be able to undergo anchorage-independent colony formation.

5.3.5. Summary

Although our investigations into the involvement of the SFKs in cellular proliferation, adhesion to fibronectin and collagen, migration, and colony forming ability do not clearly implicate a single SFK in these processes, findings from all of these studies share particular characteristics. Firstly, this work clearly demonstrates that the involvement of the SFKs in all of the cellular processes examined is cell line specific (Table 5.1 A-D). Perhaps not surprisingly, the greatest effects of both SFK inhibitor treatment and decreased Src, Fyn, and Yes expression were observed in HT29 cells, which have the highest level of Src and Yes kinase activity of the cell lines examined in these studies (Table 5.1 A). This work also demonstrates that Src, Fyn, and Yes may all be involved in a given phenotype within a single cell line. For example, the proliferation of HT29 cells was inhibited when the expression of any one of these SFKs was decreased. However, as SFK knockdown was not complete in any of the cell lines investigated, and, in some cases, was quite poor, it is likely that these proteins may be more significantly involved in given cellular phenotypes than was observed. For instance, while decreases in Src, Fyn and Yes expression resulted in phenotypic changes in HT29 and HepG2 cells, it is possible that they may also be involved in cellular processes in HCT116 and SW480 cells, or that the involvement of all three of these proteins is more significant than was observed in these studies. This may be due to the incomplete knockdown of the SFKs achieved, as the levels of Src, Fyn, and Yes remaining in our cell lines of interest following transduction with shRNA vectors or transfection with siRNAs may have been sufficient to fully carry out their functions, thereby potentially masking their involvement in certain cellular processes. The possibility of compensation by other SFKs following the loss of expression or activity of other family members may have also led to less significant effects being observed. Therefore, although decreased Src expression was found to have a greater effect on the cancer cell phenotypes investigated in this work than decreased Fyn or Yes expression, this may have been due to the more significant knockdown of Src achieved. Similarly, differences in the effects of SFK knockdown between cell lines could also potentially be due to the differing levels of Src, Fyn, and Yes knockdown in the four different cell lines examined.

Table 5.1. Summary of the effects of Src family kinase inhibitor treatment or decreased Src, Fyn, or Yes expression on the proliferation, adhesion, migration, and colony forming ability of selected cancer cell lines. A. HT29, **B.** HCT116, **C.** SW480, and **D.** HepG2 cells were examined. No statistically significant change in a phenotype following treatment or due to decreased protein expression is indicated by the – symbol; + to +++ indicates increasing levels of inhibition of the indicated cellular phenotype. Arrows indicate an increase in the cancer cell phenotype when treated with low concentrations of inhibitors, while n/a signifies that no conclusions can be drawn from the assay due to large variability.

A. HT29

	shRNA			Inhibitors			
	Src	Fyn	Yes	PP2	SU6656	SKI I	SKI II
Proliferation	++	+	++	++	–	+	–
Adhesion to Fibronectin	++	–	++	+++	+++	↑/+++	↑/–
Adhesion to Collagen	–	–	–	+++	+++	++	↑/–
Migration	+	+	+	+++	–	++	–
Colony Forming Ability	n/a	n/a	n/a	↑/+++	+++	+++	–

B. HCT116

	shRNA			Inhibitors			
	Src	Fyn	Yes	PP2	SU6656	SKI I	SKI II
Proliferation	–	–	–	++	+	–	+/–
Adhesion to Fibronectin	–	–	–	–	–	↑/–	↑/–
Adhesion to Collagen	–	–	–	++	–	↑/+	↑/+

C. SW480

	shRNA			Inhibitors			
	Src	Fyn	Yes	PP2	SU6656	SKI I	SKI II
Proliferation	++	–	–	–	–	–	–
Adhesion to Fibronectin	–	–	–	+	–	↑/–	↑/–
Adhesion to Collagen	–	–	–	++	↑/–	+	↑/–
Migration	++	–	–	+++	–	++	–

D. HepG2

	siRNA			Inhibitors			
	Src	Fyn	Yes	PP2	SU6656	SKI I	SKI II
Proliferation	–	–	–	–	–	–	–
Adhesion to Fibronectin	+	–	+	++	+	+++	–
Adhesion to Collagen	–	–	+	+	–	+	↑/–
Migration	–	–	–	++	+	+	–
Colony Forming Ability	+++	–	–	+++	+++	–	–

This work also shows that the effects of SFK inhibitor treatment on cellular processes are inhibitor specific, as the four inhibitors used in these studies were found to have different effects on multiple cancer cell phenotypes. In general, PP2 and SKI I had the greatest effects on the cellular phenotypes of all of the cell lines examined, while SU6656 also had significant effects in HT29 and HepG2 cells, and SKI II only impacted HCT116 cells (Table 5.1 A-D). In addition, the consequences of SFK inhibitor treatment were also concentration-dependent, whereby the greatest inhibition of proliferation, adhesion, migration, and colony forming ability was, not surprisingly, observed to occur when cells were treated with the highest concentration of the inhibitors. Interestingly, the adhesion of all four of our cell lines of interest to fibronectin, collagen, or both, was increased following treatment with the lowest concentration of at least one of the SFK inhibitors, as was the colony forming ability of HepG2 cells when treated with low concentrations of PP2. This highlights the importance of determining effective doses of these drugs, as they may have very different effects on certain phenotypes, depending on the concentrations being used. That the inhibitors elicited such varying responses between cell lines may also suggest that at least some of the changes in cellular phenotypes observed could be due to effects that these inhibitors have on proteins other than the SFKs. Finally, as the inhibition of the kinase activity of multiple SFKs using SFK inhibitors generally had a greater effect on all of the cellular processes examined than the individually decreased expression of Src, Fyn, or Yes, this work also suggests that multiple SFKs likely need to be targeted in order to elicit the most significant effects on cancer cells. In all, the involvement of the SFKs in various cancer cell phenotypes was shown in this work to be extremely complex.

5.4. Scope and Significance

The experiments presented in this work examined the involvement of the SFKs in cancer cell phenotypes such as proliferation, adhesion, migration, and colony formation. As the SFKs are a valid therapeutic target in a number of different cancers, it is important to investigate the involvement of these proteins in cellular processes in order to understand the consequences of their inhibition. This work clearly demonstrated that not only are multiple SFKs expressed within a cell line, but that more than one of the SFKs expressed may have kinase activity. It has also shown that the involvement of the SFKs in particular cellular processes is not clear-

cut, and, importantly, appears to be cell line specific. Furthermore, although decreased Src expression was found to result in the greatest effects on all of the cancer cell phenotypes investigated, Fyn and Yes were also implicated in certain phenotypes, suggesting that multiple SFKs may need to be targeted in order to achieve the greatest effects on cancer cells possible. In particular, and not surprisingly, targeting the SFKs in cell lines with relatively higher Src expression and activity, such as HT29 cells, was found to have a greater effect than in cells with lower Src activity, such as SW480 cells. This is important, as it suggests that the treatment of cancer cells with lower Src expression with SFK inhibitors may not be effective, and that other cancer therapies may be more useful in these cases. Also of note is the finding that the effects of SFK inhibitor treatment on the cancer cell lines investigated appear to be not only inhibitor specific, but that the effects of treatment with a given SFK inhibitor vary significantly depending on the concentration of inhibitor used. Although no clinically relevant SFK inhibitors were examined in this study, this may also potentially complicate the use of other SFK inhibitors as cancer therapies. Therefore, it will be important in the future to determine which cancers will benefit from treatment with SFK inhibitors, particularly as it is becoming increasingly evident that different cancers are best targeted by different therapeutic agents, potentially including SFK inhibitors.

6. CONCLUSIONS AND FUTURE STUDIES

An extensive body of work investigating the cellular functions of Src has accumulated since the observation of Peyton Rous that a filterable agent was capable of causing cancer in chickens nearly a century ago. More recently, it has been found that there is a whole family of similar proteins that also have important cellular functions. Significantly, the SFKs have also been implicated in a number of human diseases, including many cancers (Engen *et al*., 2008). As these proteins have been found to be involved in a variety of cancer cell phenotypes, including proliferation, adhesion, migration, angiogenesis, and metastasis, they are of significant clinical interest, and there are currently a number of SFK inhibitors in clinical trials or in use as cancer therapies (Wheeler *et al*., 2009). As such, it is important to understand the involvement of the different SFKs in cancer cell phenotypes. Although a large number of studies have examined the role of Src in these processes, far fewer investigations into the involvement of the other ubiquitously expressed SFKs, Fyn and Yes, have been carried out. This work outlines the findings that multiple SFKs are expressed in given cancer cell lines, and that the levels of protein expression and combinations of the SFKs expressed vary between cell lines. It also shows that not only Src, but also Yes and Fyn have detectable kinase activity in a subset of cancer cell lines. By examining four different cell lines using both chemical SFK inhibitors and an shRNA/siRNA approach to specifically decrease the expression of Src, Fyn, and Yes individually, this work in particular demonstrates that the effects of SFK expression are not clear-cut. Significantly, it suggests that the involvement of the SFKs in proliferation, adhesion, migration, and colony forming ability is cell line specific, and that multiple SFKs need to be targeted in order for significant effects on these cellular processes to occur.

Although this work examined the effects of decreasing the expression of Src, Fyn, or Yes individually, the level of knockdown achieved was less than ideal and may have prevented the involvement of these proteins in cancer cell phenotypes from being observed. It would also have been useful to examine the effects of knocking down different combinations of SFKs in order to more fully understand the involvement of these proteins in various cellular processes. Such studies could be used to determine if multiple SFKs are involved in given cancer cell

phenotypes, and if they have complementary and/or compensatory functions. Furthermore, cells in which the expression of specific combinations of SFKs were decreased would provide an important tool that could be used to determine if the effects of SFK inhibitors on cancer cells are due to the inhibition of these proteins or are instead due in part to non-specific effects on other proteins. This is likely the case, as many of the SFK inhibitors currently in clinical trials target other proteins, in particular Abl kinase, in addition to the SFKs (Wheeler *et al*., 2009). As Src and the other SFKs are likely to remain attractive targets in cancer therapy in the future, it is important to understand their functions in cancer cells.

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